Journal of Supramolecular Structure
and Cellular Biochemistry 15:253–286 (1981)
Control of Cellular Division and Development: Part A: 411–444

Multiplication Stimulating Activity (MSA) From the BRL 3A Rat Liver Cell Line: Relation to Human Somatomedins and Insulin

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The properties of multiplication stimulating activity (MSA), an insulin-like growth factor (somatomedin) purified from culture medium conditioned by the BRL 3A rat liver cell line are summarized. The relationship of MSA to somatomedins purified from human and rat plasma are considered. MSA appears to be the predominant somatomedin in fetal rat serum, but a minor component of adult rat somatomedin. In vitro biological effects of MSA and insulin in adipocytes, fibroblasts and chondrocytes are examined to determine whether they are mediated by insulin receptors or insulin-like growth factor receptors. The possible relationship of a primary defect of insulin receptors observed in fibroblasts from a patient with the rare genetic disorder, leprechaunism, to intrauterine growth retardation is discussed.

Key words: insulin-like growth factor, somatomedin, multiplication stimulating activitv, insulin, receptors: insulin; insulin-like growth factor, fibroblasts, DNA synthesis, amino acid transport, glucose, leprechaunism, liver cells

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Received June 6, 1980; accepted September 15, 1980.

Abbreviations: MSA, multiplication stimulating activity; IGF, insulin-like growth factor; NSILA-s, acid ethanol soluble nonsuppressible insulin-like activity; BRL, Buffalo rat liver; NGF, nerve growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; hypox, hypophysectomized; GH, growth hormone; LDL, low-density lipoprotein; AIB, α-aminoisobutyric acid; TAT, tyrosine aminotransferase; LH, luteinizing hormone; FSH, follicle stimulating hormone; PGE_1, prostaglandin E_1.

0275-3723/81/1503-0253$09.00

MULTIPLICATION STIMULATING ACTIVITY (MSA): AN INSULIN-LIKE GROWTH FACTOR FROM A RAT LIVER CELL LINE

Definitions

Multiplication-stimulating activity (MSA) is the name given by Dulak and Temin [1] to a family of closely related polypeptides synthesized by a particular line of cells (BRL 3A) derived from normal rat liver. Our laboratories have purified two forms of MSA to homogeneity and have characterized their chemical, biological, and immunological properties and their interactions with specific cell surface receptors and serum carrier proteins [2–5]. It became apparent that, as postulated by Dulak and Temin, MSA from BRL 3A cells (BRL-MSA) closely resembled peptides present in human plasma known as somatomedins [6, 7] and nonsuppressible insulin-like activity [8, 9]. Direct comparative studies of MSA and homogeneous preparations of the human peptides (insulin-like growth factor (IGF)I, IGF-II, somatomedin A, and somatomedin C) have established that these five peptides are a family of closely related molecules [10–12]. We shall refer to this group of peptides as insulin-like growth factors because the two members of the group whose amino acid sequences have been defined, IGF-I and IGF-II, show remarkable sequence identity with insulin [13, 14]. The human peptides appear to differ in the extent to which their plasma levels are regulated by growth hormone, suggesting that only some members of the group may be mediators of growth hormone action or somatomedins [6, 7, 9, 15].

The properties common to the 5 insulin-like growth factors are summarized in Table I. Two features deserve special comment: 1) The extraordinary structural similarity – IGF-I and IGF-II are identical at 62% of their amino acid positions – and 2) preservation of the molecular features most related to biological function – recognition by cell surface receptors and by serum carrier proteins.

The designation insulin-related growth factor also has been used recently by Bradshaw and Niall [16, 17] to refer to a partially overlapping set of polypeptides, IGF-I, IGF-II, NGF, and relaxin [18, 19], to call attention to structural similarities and a presumed evolutionary relationship. It should be noted that IGF-I is much more closely related to IGF-II (≅75% identical residues in the regions corresponding to insulin A and B chains) than to either NGF (14% identity) or relaxin (26% identity). Moreover, as summarized in Table II, IGF-I, NGF, and relaxin are synthesized by different tissues and have different biological activities in different target organs. Although relaxin stimulated thymidine incorporation in human fibroblasts [23] like insulin and IGFs [2, 10], relaxin from Dr. C. Schwabe [21] did not stimulate thymidine incorporation in chick embryo fibroblasts [S.P. Nissley, unpublished results]. Whereas IGF-I and insulin reacted with IGF receptors and insulin receptors, NGF and relaxin did not cross-react with these receptors, nor did insulin and IGF-I cross-react with relaxin and NGF receptors (Table III). In addition, NGF did not react with antibodies to MSA [32]. Thus, our classification of insulin-like growth factors emphasizes a functional relationship (probably based on close sequence homology), whereas the alternate classification of insulin-related growth factors that includes NGF and relaxin [16, 17], emphasizes a more distant chemical and possible evolutionary relationship.

Properties of MSA Purified From BRL 3A Conditioned Medium at NIH

The BRL 3A cell line was established in culture by Hayden Coon in 1968 by primary cloning from the liver of a 5-week-old normal rat [33]. It had the interesting prop-

TABLE I. Common Properties of the Insulin-Like Growth Factors (IGF-I, IGF-II, Somatomedin A, Somatomedin C, MSA)*

A. Chemical
   1. Single-chain polypeptides
   2. Molecular weight 7,000–9,000
   3. Acid soluble
   4. Acid and heat stable
   5. Inactivated by thiols[a]
   6. Amino acid sequence homology:
      a. IGF-I and IGF-II identical at 45 of 73 positions (62%)
      b. Somatomedin C identical to IGF-I at 22 of 25 positions tested; however, 2 fragments (8 and 9 residues) in somatomedin C are not present in IGF-I

B. Biological
   1. Adipose tissue: insulin-like activity (eg, glucose oxidation, lipogenesis); 0.2–2.0% as potent as insulin; not inhibited by antibodies to insulin
   2. Cartilage: sulfate incorporation into glycosaminoglycans; DNA, RNA, protein synthesis; hypox rat costal cartilage, chick pelvic leaflet
   3. Chick embryo fibroblasts in culture: DNA synthesis, cell multiplication

C. Occurrence
   Present in human plasma (IGF-I, IGF-II, somatomedin A, somatomedin C), rat plasma (rat somatomedin, MSA-like peptides), and BRL 3A conditioned medium (MSA) in association with higher molecular weight carrier proteins. Binding protein-IGF complexes dissociate at acid pH.

D. Recognition by specific receptors, binding proteins, and antibodies
   1. Receptors
      a. Insulin receptors: weak cross-reaction
      b. Insulin-like growth factor receptors
   2. Carrier proteins from rat and human serum
   3. Antibodies

*Based on references 2, 6, 7, and 9.
[a]Not reported for somatomedin A.

TABLE II. Properties of Peptides Structurally Related to Insulin

	Insulin [20]	IGF [9]	Relaxin [16, 21]	NGF [22]
1. Tissue source	Pancreas (β-cell)	Plasma Liver Liver-derived cells	Ovary (pregnant corpus luteum)	Submaxillary gland (male mouse)
2. Target tissues	Fat Muscle Liver	Cartilage Muscle	Pubic symphysis Uterus	Sensory and sympathetic neurons
3. Biological activities	Glucose transport and metabolism Amino acid transport Lipogenesis Gluconeogenesis Cell growth	Pleiotypic anabolic effects in cartilage, fibroblasts, muscle	Dilate cervix Inhibit uterine contractions Relax pubic ligaments	Development and survival of neurons

TABLE III. Cross-Reactivity of Peptides Structurally Related to Insulin in Receptor Assays

	Insulin	IGF	Relaxin	NGF
^{125}I-insulin	+	+ (weak)	– [23]	– [28]
^{125}I-MSA (IGF)	+ (some)	+	– [a,b]	– [c]
^{125}I-relaxin	– [23] [d]	+ (weak) [23a]	+ [23]	NT [e]
^{125}I-NGF	+ [29, 30] [f], – [28, 31]	–	–	+

[a] Relaxin [18]: rat liver plasma membranes [M.M. Rechler, unpublished results].
[b] Relaxin [19]: chick embryo fibroblasts [M. M. Rechler, unpublished results].
[c] Chick embryo fibroblasts [24]; human skin fibroblasts [25]; rat liver plasma membranes [M. M. Rechler, unpublished results]; human placental membranes [26]; NRK fibroblasts [27].
[d] Weak inhibition by proinsulin in human skin fibroblasts [23] and rat uterus [23a].
[e] NT = not tested.
[f] < 30% inhibition: chick embryo: dorsal root and sympathetic ganglia [29]; dissociated heart and brain [30].

erty that, following plating at low density in medium containing serum, BRL 3A cells would replicate at a nearly normal rate for several generations in serum-free medium without protein or hormonal supplements. This cell line was used by Dulak and Temin [1] for the initial isolation of MSA. We obtained BRL 3A cells from Dr. Temin in 1973. The BRL 3A cell line was submitted to the American Type Culture Collection in 1978 for distribution. Its ATCC designation is CRL 1442.

The starting material for MSA isolation is serum-free medium conditioned by confluent cultures of BRL 3A cells for 3–4 days. The purification scheme in effect in our laboratories from 1976 to 1980 has recently been published [34]. In brief, conditioned medium is chromatographed on Dowex 50 X-8. The pH 11 eluate containing MSA is chromatographed on Sephadex G-75. Fractions containing MSA activity (ie, capable of stimulating the incorporation of [^{3}H]thymidine into the DNA of serum-starved tertiary passage chick embryo fibroblasts) were further analyzed by disc gel electrophoresis at pH 2.7 in 9 M urea. Based on the electrophoretic pattern, fractions from the G-75 column have been grouped into 3 broad regions: peak I, peak II, and peak III. Peak I contains a single MSA species, mol wt 16,300, R_f 0.36; peak II contains 4 closely migrating species, mol wt 8,700, R_f 0.41–0.49; peak III contains 2 closely migrating species, mol wt 7,100, R_f 0.59–0.62.

Two species of MSA have been isolated in apparently homogeneous form from pools of the appropriate Sephadex G-75 fractions by preparative acrylamide gel electrophoresis under conditions similar to those of the analytical gel electrophoresis. These peptides have been designated MSA II-1 (mol wt 8,700) and MSA III-2 (mol wt 7,100).* MSA II-1 gives a single protein band following electrophoresis at two pHs and has a single COOH-terminal amino acid (glycine) [34]. Although MSA II-1 has a lower specific insulin-like activity than IGF-I in stimulating glucose oxidation and lipogenesis in adipocytes (50 mU/mg versus 144–170 mU/mg), this may represent the intrinsic potency of MSA II-1 rather than heterogeneity of the preparation [24, 35, 36]. MSA II-1 has been radioiodinated [24] and used as ligand in receptor [24] assays, binding protein [38, 39] assays, and some immunoassays [32]. MSA III-2 has been radioiodinated and used as ligand in some immunoassays [32], since it appears to bind preferentially to a different antibody population in one immune rabbit serum.

*These molecular weight assignments are based on Sepharose 6B-guanidine chromatography. They are considered provisional pending determination of the amino acid sequence of MSA II-1 and MSA III-2. For this reason, MSA potencies are expressed in ng/ml rather than in molar concentrations.

Sephadex G-75 peak II pool has been substituted for MSA II-1 in many biological studies and as standard in competitive binding assays. The rationale for this substitution is as follows. The G-75 peak II pool contains only 4 protein species on analytical gel electrophoresis [34]. The mixture of peak II peptides and MSA II-1 possess identical activity in the chick fibroblast bioassay (Fig. 1), radioimmunoassay (Fig. 1), and rat liver membrane receptor assay (unpublished results). The amino acid compositions of Sephadex peak II MSA, homogeneous MSA II-1, and a mixture of MSA II-2, 3, 4 obtained following preparative electrophoresis are indistinguishable [34]. The molecular weights of MSA II-1 and MSA II-2, 3, 4 determined by gel filtration on Sepharose 6B in 6 M guanidine HCl (in the absence of reducing agents) were identical, 8,700 [34]. In fact, the only discernible difference (aside from minor differences in electrophoretic mobility) between MSA II-1 and MSA II-2, 3, 4 occurred following incubation with 6 M guanidine HCl in the presence of reducing agents: The molecular weight of MSA II-1 was unaffected, whereas a mixture of MSA II-2, 3, 4 peptides was partially converted to lower molecular weight forms [34]. We interpret these results to indicate that some forms of MSA II-2, 3, 4 contain interruptions in the polypeptide chain but that 1) this break does not occur at a site critical for biological activity or recognition by receptors, antibody, or binding protein, and 2) in the absence of reducing agents, these cleaved molecules are held together in the native conformation by disulfide bonds.

Considerable evidence suggests that the sequence of MSA III-2 is contained within MSA II-1 [32, 34]. No amino acids are significantly more abundant in MSA III-2 than in

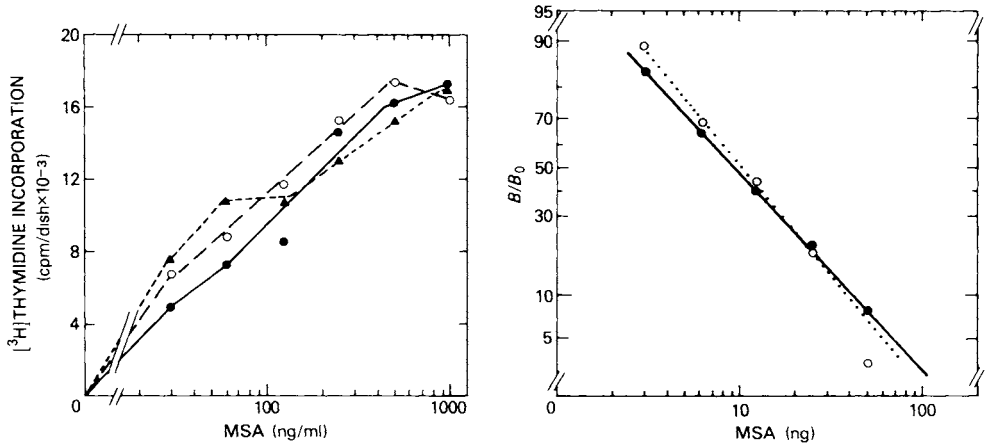

Fig. 1. Left: stimulation of [^{3}H]thymidine incorporation into DNA of serum-starved tertiary chick embryo fibroblasts by different MSA preparations: Sephadex G-75 peak II (•), MSA II-1 (○), and a mixture of MSA II-2, -3, and -4 (▲) obtained by preparative electrophoresis. MSA preparations were purified as previously described [32]. Confluent fibroblast cultures in 60 mm dishes were plated in Temin's modified Eagle's medium containing 0.4% calf serum. After 3–5 days, peptides were added in medium without serum for 12 h. The cultures were then pulsed for 1 h with [^{3}H]thymidine, and the incorporation of radioactivity into acid-precipitable material was quantitated as previously described [40]. Redrawn from [34] with permission.

Right: reactivity of MSA peptides in MSA radioimmunoassay. The immunoassay was performed as previously described [32], with MSA antiserum at 1:1,000 dilution, ^{125}I-labeled MSA II-1, and precipitation of antibody-bound radioactivity with polyethylene glycol. Dilutions of MSA II-1 (•) and a mixture of MSA II-3 and -4 (○) were tested. B_0 is the radioactivity bound to antibody in the absence of unlabeled MSA; B is the radioactivity bound at each concentration of unlabeled MSA. Results are plotted on a logit-log scale. Redrawn from [32] with permission.

MSA II-1. Antibodies raised in rabbits to Sephadex G-75 MSA peak II (and devoid of MSA III-2) recognize MSA III-2. In fact, in a radioimmunoassay using ^{125}I-labeled MSA III-2, parallel dose-response curves are obtained with MSA III-2, MSA II-1, and peak I MSA, suggesting the presence of a common antigenic determinant. However, using ^{125}I-labeled MSA II-1 and the same antiserum, MSA III-2 competes poorly, with a dose-response curve markedly nonparallel to the MSA II-1 standard. MSA III-2 does not react at all with a different antiserum to peak II MSA [J. A. Romanus and M. M. Rechler, unpublished results]. This suggests that these populations of antibodies to peak II MSA recognize determinants that have been lost or altered in MSA III-2.

MSA III-2 reacts with MSA receptors and with the MSA binding protein of rat serum. It is approximately 3-fold more potent than MSA II-1 against the rat liver membrane receptor [4], chick embryo fibroblast receptor [unpublished results, M.M. Rechler and J.A. Romanus], somatomedin binding protein [4], and the insulin receptor of placental membranes [41]. Peak III MSA is more potent than peak II MSA in stimulating DNA synthesis (Fig. 2). When ^{125}I-MSA II-1 was incubated with chick embryo fibroblasts for 18 h under conditions of the thymidine incorporation bioassay, no conversion to MSA III-2 could be detected by gel filtration on Sephadex G-75 in 1 M acetic acid [Y. Yang and M. M. Rechler, unpublished results]. Reduction of MSA II-3 generated a species electrophoretically indistinguishable from MSA III-2 [34].

MSA peak I has not been purified to homogeneity and has been less extensively studied. Peak I possesses MSA biological activity (Fig. 2), and following electrophoresis, this activity corresponds to the major protein band in the preparation [34]. Peak I MSA reacts with the MSA receptor of liver membranes [34], weakly reacts with rat serum

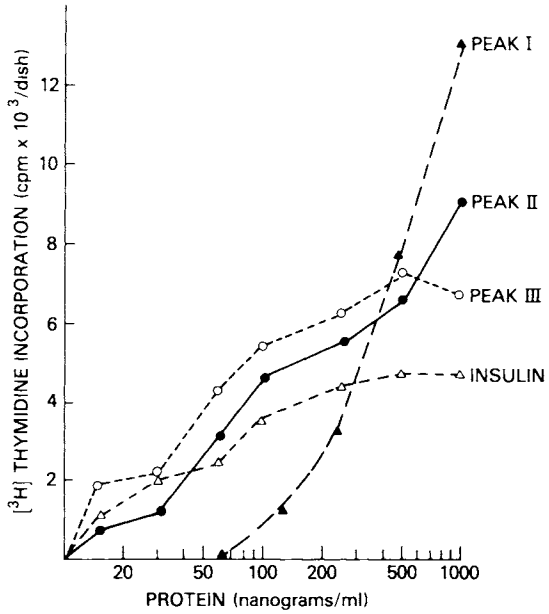

Fig. 2. Activity of different Sephadex G-75 purified MSA fractions and insulin in the chick embryo fibroblast bioassay. MSA fractions were purified as described elsewhere [34]. Porcine insulin was purchased from Eli Lilly Co. Conditions for [^{3}H]thymidine incorporation were as described in Figure 1 (left).

binding protein [4], and gives a displacement curve parallel to MSA III-2 and MSA II-1 in the radioimmunoassay using ^{125}I-MSA III-2 [32]. Peak I MSA may represent a precursor form.

Assays for MSA

Several assays for MSA are commonly in use in our laboratory. Their sensitivity and specificity for MSA species, human somatomedins, rat serum somatomedins, and insulin are summarized in Table IV. No one assay measures a single MSA-related polypeptide without cross-reaction of the other species. Interpretation of results with complex mixtures should be made with caution until the relative contributions of different reacting species can be resolved.

Relation of NIH-MSA to Other Known MSA Preparations From the BRL 3A Cell Line

Dulak MSA. MSA purified by Dowex chromatography and successive preparative electrophoreses in SDS and acetic acid-urea as described by Dulak and Shing [47] was kindly provided by Dr. Dulak. Dulak-MSA was equipotent with NIH-MSA peak II in the thymidine incorporation bioassay, rat liver membrane receptor assay, and radioimmunoassay [S. P. Nissley, M. M. Rechler, and A. C. Moses, unpublished results]. Gel electrophoresis at both acid and alkaline pHs [34] identified the major component in Dulak MSA as II-1, with other peak II peptides (but no non-MSA peptides) represented.

Collaborative Research. Partially purified MSA (after Sephadex G-50 gel filtration in 1 M acetic acid) was provided by R. Forand and tested in April 1978. Two components were found to have 50–100% the potency of NIH-MSA peak II in the chick embryo fibroblast bioassay [S.P. Nissley, unpublished results]. We have not examined the electrophoretic purity of this preparation.

New Zealand MSA. Brinsmead and Liggins [26] have purified MSA by Dowex 50 and Sephadex G-50 chromatography. In comparative studies, NIH-MSA and New Zealand-MSA were within a factor of 2 in potency in 1) the placental membrane receptor assay using ^{125}I-MSA [26] and 2) the chick embryo fibroblast thymidine incorporation bioassay [S.P. Nissley, unpublished results]. We have not performed electrophoretic studies with the New Zealand MSA preparation.

Relationship of MSA to Homogeneous Human Insulin-Like Growth Factors

MSA has been extensively compared with somatomedin A [10] and with IGF-I and IGF-II [11, 12, 24]. In competitive binding assays using multiple receptor systems or rat serum binding proteins, results obtained with a matrix of ^{125}I-labeled peptides and unlabeled peptides uniformly indicated substantial cross-reactivity [10, 11], although minor differences were appreciated in the relative potencies of different peptide preparations. For example, the results in Figure 3 suggest that ^{125}I-MSA and ^{125}I-IGF-I may, under appropriate conditions, bind to the same receptor in human fibroblasts, identified by the relative potencies of the competing unlabeled peptides. The 4 peptides also manifested similar biological activities; for example, they stimulated lipogenesis in rat fat pad [35] and DNA synthesis in chick embryo fibroblasts [10, 35].

Immunological differences between MSA and the human somatomedins are much greater than the differences in biological activity and in reactivity with receptors and binding proteins. MSA II-1 did not react in immunoassays for IGF-I [9], IGF-II [9], and somatomedin A [48] at concentrations indicating potencies <2%. Somewhat greater

TABLE IV. Assays for MSA in Use in Our Laboratories

Assay	Bioassay: [^{3}H]thymidine incorporation, chick embryo fibroblasts	Receptor assay: rat liver plasma membranes	Radioimmunoassay [32]: ^{125}I-MSA III-2	Radioimmunoassay [32]: ^{125}I-MSA II-1	Competitive binding assay: binding proteins[a]
1) Sensitivity (ED_{50})	≅30 ng/ml	≅8 ng/ml	≅ 4 ng/ml	≅13 ng/ml	≅10 ng/ml[b]
2) Specificity					
MSA species	III > II > I (Fig. 2)	III > II > I [4]	III > II > I	II > I; III not parallel	III > II > I [4]
Insulin	+ (Fig. 2) [42]	– [42]	–	–	–
Proinsulin	+ [43][c]	–[d]	NT[e]	–	NT
IGF-I, IGF-II	+ [45]	+ [12][f]	+[g]	Not parallel, weak	+ [11, 39]
Somatomedin A	+ [10]	+ [11][f]	+	NT	+ [39]
Somatomedin C	+ [44]	+ [10]	NT	NT	±[d]
Rat somatomedin	+ (Table V)	+ (Fig. 4)	– (Table V)	NT	+ (Table V)
3) Rat serum	Hypox[h] < normal [4] Hypox < GH-treated hypox [4] Maternal ≅ fetal [4]	– (Table V) Fetal high [46]	Hypox < normal [4] Fetal >> maternal [46]	NT	Fetal high [46]

[a]Normal rat serum, GIBCO.

[b]Sensitivity when bound and free ^{125}I-MSA were separated using charcoal activated with fatty acid-free albumin. Approximately 5–10-fold lower sensitivity was observed with other albumin preparations [39].

[c]Proinsulin 20% as potent as insulin.

[d]Unpublished results, M. M. Rechler.

[e]NT = Not tested.

[f]IGF-II most potent.

[g]MSA II-1:IGF-II:IGF-I = 100:10:3 (parallel).

[h]hypox = hypophysectomized; GH = growth hormone.

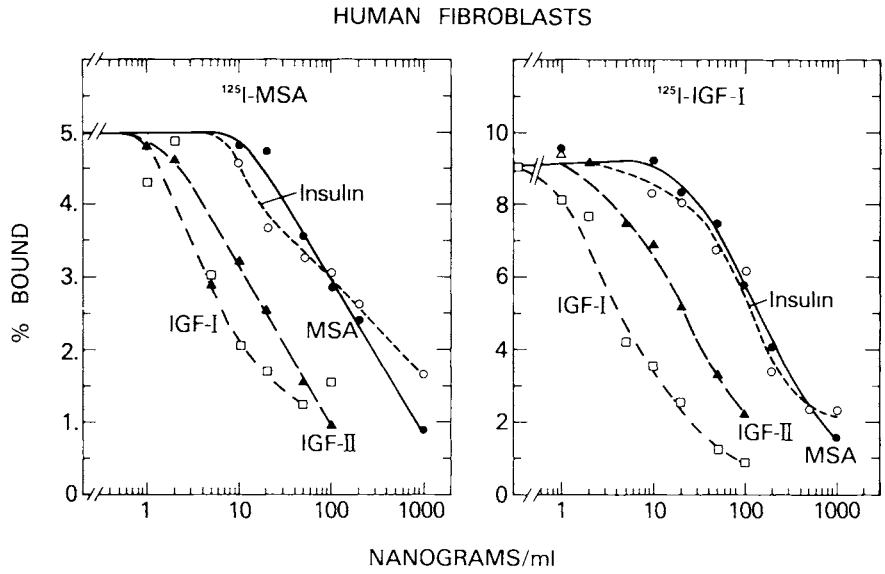

Fig 3. Competition for binding of ^{125}I-labeled MSA (left) and ^{125}I-labeled IGF-I (right) to human skin fibroblasts in culture by unlabeled IGF-I and IGF-II (a kind gift of Dr. R. E. Humbel), Sephadex G-75 peak II MSA, and porcine insulin Conditions of the binding assay have been previously described [11, 25]. Percent of input radioactivity bound to 1.3–1.5 × 10^6 suspended fibroblasts following 2 h incubation at 15°C is shown

The results presented for ^{125}I-IGF-I are observed consistently, with the exception that the MSA dose-response curve is typically to the left of the insulin curve The curve presented for unlabeled IGF-I inhibition of ^{125}I-MSA binding is atypical in one respect In other experiments, unlabeled IGF-I only inhibited specific binding by ≅50%, reaching a plateau [11] The latter result has been interpreted as indicating that ^{125}I-MSA binds to two sites in human fibroblasts, only one of which interacts with IGF-I Apparently cell culture conditions affect the relative proportions of the two sites, in the present experiment, ^{125}I-MSA binds predominantly to the IGF-I inhibitable site, which presumably is identical to the ^{125}I-IGF-I binding site

cross-reactivity was observed in the MSA radioimmunoassay using ^{125}I-MSA III-2; IGF-II and IGF-I were 10% and 3% as potent as MSA II-1 [32].

Recently, homogeneous preparations of somatomedin C [49] have been kindly provided by Dr. Van Wyk and compared with MSA. ^{125}I-somatomedin C appeared to bind to the same receptor as ^{125}I-MSA in chick embryo and human fibroblast cultures, but not in liver membranes or cultured liver cells. ^{125}I-somatomedin C (and ^{125}I-MSA) binding to chick embryo fibroblasts and human fibroblasts was inhibited by unlabeled MSA, somatomedin A, and insulin [M. M. Rechler and J. M. Podskalny, unpublished results]. Binding of ^{125}I-somatomedin C to BRL 3A2 cells (a subclone of BRL 3A) also was inhibited by insulin (nonparallel), as well as by MSA and somatomedin A [unpublished results]. By contrast, binding of ^{125}I-MSA and ^{125}I-IGF-II to BRL 3A2 cells was not inhibited by insulin [2, 11], whereas ^{125}I-IGF-I and ^{125}I-somatomedin A were similar to ^{125}I-somatomedin C in this respect [10, 11]. Like ^{125}I-somatomedin A [10] and ^{125}I-IGF-I [11], ^{125}I-somatomedin C bound poorly to purified rat liver plasma membranes [unpublished results]. However, unlabeled somatomedin C, MSA, and somatomedin A potently competed for ^{125}I-MSA binding to liver membranes (Fig. 4). This suggests differences between the iodo- and non-iodopeptides. In the placental membrane assay using ^{125}I-somatomedin C, MSA III-2 and MSA II-1

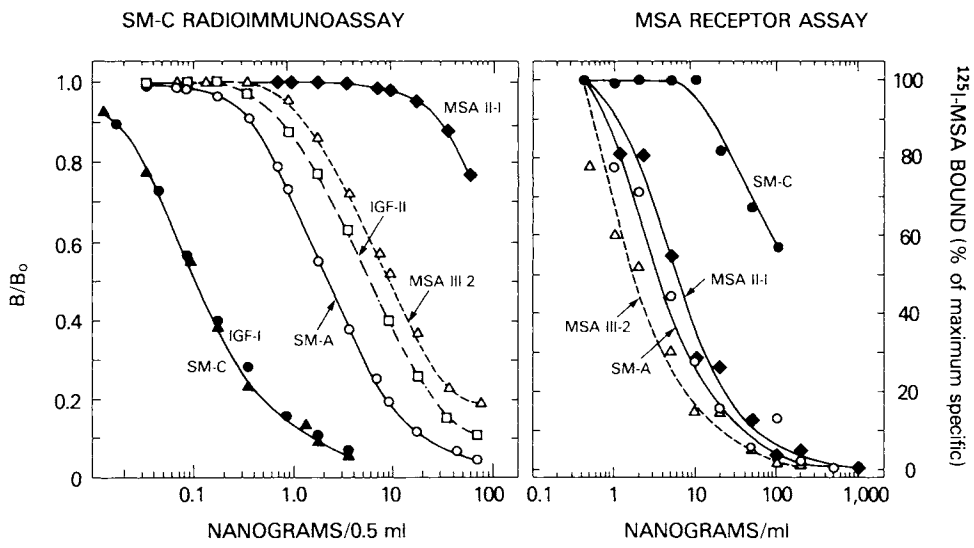

Fig. 4. Left: cross-reactivity of MSA III-2 and MSA II-1 in the radioimmunoassay for somatomedin C, using rabbit anti-human somatomedin C [50]. The ordinate represents binding (B) of ^{125}I-somatomedin C, expressed relative to maximum tracer binding in the absence of competing unlabeled peptide (B_0). Redrawn from [41] with permission.

Right: cross-reactivity of somatomedin C (•) and somatomedin A (○) in the radioreceptor assay using rat liver plasma membranes and ^{125}I-labeled MSA II-1. Assay conditions were as previously described [10, 24]. The same preparations of somatomedin C, MSA III-2, and MSA II-1 were used as in the left-hand panel. Homogeneous somatomedin A (SPE 2451) was a kind gift of Dr. Linda Fryklund (AB KABI, Stockholm). The percent of ^{125}I-MSA bound (equal to 10.7% of input radioactivity) is plotted against ng/ml of unlabeled peptide in the incubation.

were 21% and 5% as potent as unlabeled somatomedin C, respectively [41]. Finally, MSA III-2 was 1% as potent as somatomedin C in the somatomedin C radioimmunoassay (Fig. 4); IGF-II and somatomedin A were 2- and 4-fold more potent than MSA III-2, whereas MSA II-1 was 10-fold less potent [41].

This completes the demonstration that BRL-MSA is closely related to each of the defined members of the family of human somatomedins or insulin-like growth factors. Undoubtedly, this relatedness is based on major structural identities. IGF-I and IGF-II are identical at >60% of their residues [13, 14]. Partial sequence data on somatomedin C indicate that it, too, is quite similar to IGF-I [49], and IGF-I and somatomedin C are indistinguishable in radioreceptor and radioimmunoassays [41]. Residues at 22 of 25 sites identified in somatomedin C (9 NH_2-terminal residues and 3 internal tryptic peptides) correspond to residues in IGF-I [49]. However, two additional tryptic peptides containing 8 and 9 residues, respectively, do not match known IGF-I sequences, suggesting that the two molecules are similar but not identical [49].

Although it is not yet possible to decide which human somatomedin/IGF MSA resembles most closely, MSA and iodinated MSA serve as useful surrogates for the group of human somatomedins. The IGF/somatomedins and the cell surface receptors with which these peptides interact, however, are heterogeneous. To define completely how somatomedins interact with a given target tissue will require use of a panel of purified IGFs, including MSA. Hopefully, the subtle differences in reactivity will soon become understood in terms of the chemical and structural differences among this family of similar peptides.

TABLE V. Reactivity of Partially Purified Rat Somatomedin in Bioassays and Competitive Binding Assays for MSA

Assay	Potency of rat somatomedins relative to MSA peak II (%)[a]
1) Bioassay: [^{3}H]thymidine incorporation, chick embryo fibroblasts	≈60
2) Receptor assay: rat liver membranes	<0.1[b]
3) Receptor assay: chick embryo fibroblasts	≈10[c]
4) Competitive binding assay using rat serum binding proteins	≈3
5) Radioimmunoassay[d]	<0.25[d]

[a]Rat somatomedin was purified 40,000-fold from the serum of rats bearing the growth hormone-secreting tumor MStT/W15 by hollow fiber ultrafiltration, gel filtration, isoelectric focusing, and cellulose thin-layer chromatography (2 times) [53] Rat somatomedin protein content was estimated from amino acid analysis. Its activity was 86 somatomedin units/mg protein in the hypox rat costal cartilage bioassay, and ≈46 milliunits insulin-like activity/mg protein in the placental membrane receptor assay using IGF-I tracer and partially purified NSILA standard [I. Mariz and W. H. Daughaday, personal communication]. In contrast to less purified preparations, this preparation was not contaminated by peptides resembling the C_{3A} component of complement, a serum protein that copurified with rat somatomedin in early stages of purification [52]. The purity of this preparation is not known. Experiments shown were performed with the same rat somatomedin preparation (Amicon 10–16).
[b]Inhibition of ≈25% only at highest concentration tested.
[c]Estimated. Rat somatomedin was 3.2% as potent as MSA III-2 in this experiment. In other experiments, MSA III-2 is approximately 3-fold more potent than MSA II-1.
[d]Radioimmunoassay used MSA III-2 tracer No inhibition by rat somatomedin at highest concentration tested (400 ng).

Relation of MSA to Somatomedin in Rat Serum

Whether MSA synthesized by the BRL 3A rat liver cell line corresponds to a somatomedin that circulates in rat plasma cannot be definitively answered until the amino acid sequences of MSA and the rat somatomedins are available. Present information suggests that MSA represents a minor component of the somatomedins in adult rat plasma, but it may be the predominant somatomedin in fetal rats.

1) Radioimmunoassay highly specific for MSA indicated that 50–100 ng/ml of MSA equivalent are present in normal adult rat serum. By contrast, bioassay (thymidine incorporation in chick embryo fibroblasts) yields values of approximately 5 μg/ml MSA in rat serum [4]. Our provisional conclusion is that rat serum contains other low molecular weight, acid-soluble, biologically active, growth hormone-dependent peptides – presumably somatomedins – that do not react with antibodies to MSA. That is, MSA (by immunologic criteria) would represent a minor fraction of the somatomedin activity in normal adult rat serum. Two qualifications of this interpretation should be made: First, all of the biologic activity in rat serum may not result from somatomedins, and second, non-somatomedin components in rat serum may act synergistically with MSA to increase its growth-stimulating effect on chick embryo fibroblasts. For example, Cohen and Nissley have described enhancement of the mitogenic effect of MSA by Cohn Fraction VI of human serum [51].

2) A partially purified preparation of rat somatomedin has been obtained from the serum of rats bearing a growth hormone-producing tumor [52, 53]. Preparations have been made available for comparison with MSA by W. H. Daughaday and I. Mariz. As summarized in Table V, rat somatomedin had considerable activity in the MSA bioassay,

weak but distinct reactivity with chick fibroblast MSA receptors and rat serum somatomedin binding protein, and negligible reactivity with rat liver membrane MSA receptors and MSA antibodies.

Thus, the rat somatomedin preparation tested shows significant reactivity in three MSA assays, indicating that it contains one or more polypeptides related to MSA. The lack of reactivity in the MSA immunoassay and liver membrane receptor assay suggests that these related peptides, however, are not identical with BRL-MSA. The relationship of this rat somatomedin preparation obtained from a tumor-bearing animal to the somatomedins present in normal rat serum remains to be established.

MSA RECEPTORS AND INSULIN RECEPTORS: MEDIATION OF BIOLOGICAL RESPONSES

Introduction

The underlying assumption and working hypothesis during the last decade of research on polypeptide hormone receptors is that the hormones bind to specific receptors on the cell surface, and that a series of biochemical events are triggered by the binding reaction that result in a biological response [54]. This simple statement has been complicated by the realization that some polypeptides are taken into the cell (eg, EGF), often with their receptors [55], and that receptors for peptide hormones are present on membranes of internal organelles [56]. The functional significance of these latter observations is not yet fully understood: whether they are part of the transmission of the signal, or, alternatively, a means to terminate the signal, to regulate receptor number, or a stage in the synthesis of new membrane receptors. As a first approximation, it seems reasonable that binding to cell surface receptors is the first step in peptide hormone action, and that the specificity of the binding reaction should mirror that of biological potency at least semi-quantitatively. We shall briefly review the evidence that separate specific receptors occur on most cells for the insulin-like growth factors and for insulin and that these receptors (like the hormones themselves) have certain similarities; namely, they cross-react with the same spectrum of peptides. From the specificity of binding and biological responses, we shall attempt to deduce which receptor mediates particular biological effects.

An operational definition of an insulin receptor is that it binds ^{125}I-labeled insulin and that the relative abilities of other peptides to bind to this receptor (measured as competition for binding of radioligand) reflect the insulin-like biological potency of these molecules [54]. A panel of insulins of different biological potencies might include insulins of different species (pig, fish, chicken, guinea pig), chemically or enzymatically modified insulins (desalanine-desasparagine insulin, desoctapeptide insulin), and insulin precursors (proinsulin). If ^{125}I-insulin binds to the biologically relevant site, and if all that is required of the hormone to initiate a biological response is to bind to the receptor, then one would expect that the order and approximate potencies of these peptides should be about the same for biological effect and inhibition of binding. Given the complexities of the assay systems and the many unknown components, it would seem fortuitous if the potencies for biological effect and binding correspond quantitatively. Strict correspondence might not occur if 1) the receptor population binding a radioligand were heterogeneous; receptors may have different affinities, the same affinity but different specificity, or the same affinity and specificity but be connected to different biological functions (functional compartmentalization); 2) spare receptors were present; 3) hormones were degraded at different rates; 4) steps subsequent to binding and involved in signal transmission (eg, internalization, binding to intracellular sites) required active participation of the hormone.

The insulin-like growth factors have weak insulin-like activity in adipocytes that is not inhibited by antibodies to insulin [8]. Hintz et al [57] initially demonstrated that somatomedins weakly inhibited binding of ^{125}I-insulin to the insulin receptors of placental membranes. This observation has been extended to the other human somatomedins and MSA [2, 9, 58, 59]. In general, the insulin-like growth factors are 100–1,000-fold less potent than insulin in biological and binding assays for insulin.

Specific receptors for insulin-like growth factors soon were demonstrated in placenta [58, 59], chick embryo fibroblasts [24, 60], rat liver plasma membranes [42, 61, 62], and thereafter in a broad range of tissues and cultured cells [reviewed in 2, 12]. These receptors interact with all other insulin-like growth factors (Figs. 3, 4). They do not react with chemically unrelated peptides (eg, EGF, hGH, glucagon) or functionally distinct peptides (eg, relaxin and NGF; see Table III). IGF receptors differ in their reactivity with insulin (and proinsulin). The MSA receptor in rat liver plasma membranes is <0.1% as reactive with insulin and proinsulin as with MSA [24, 42] (Table IV).* By contrast, insulin reacts with the MSA receptor of chick embryo fibroblasts quite effectively, being ≅50% as potent as MSA [24]. Insulin reacts with the IGF-I/MSA receptor of cultured human fibroblasts 2–10% as potently as unlabeled IGF-I and is comparable in potency to unlabeled MSA (Fig. 3). Intermediate reactivity was observed in placenta; insulin is 0.1–1% as potent as the insulin-like growth factors in inhibiting radiolabeled IGF binding to placental membranes [26, 58, 59].

The MSA receptors of human fibroblasts [25] appear to share immunologic determinants with insulin receptors, indicating a possible structural relationship between the two receptors [12, 64]. This was studied using IgG purified from the serum of a patient with the type B syndrome of extreme insulin-resistant diabetes mellitus and acanthosis nigricans [65, 66], in which circulating antibodies to insulin receptors have been demonstrated. Antireceptor IgG inhibits binding of ^{125}I-insulin to insulin receptors of rat liver membranes and human fibroblasts (Fig. 5). The inhibition of binding is quite specific: IgG from controls did not inhibit insulin binding, and the patient's IgG did not inhibit the binding of the chemically unrelated polypeptides EGF and glucagon. Concentrations of antireceptor IgG that inhibited binding of ^{125}I-insulin by 75–95% in liver membranes inhibited ^{125}I-MSA binding by only 5% and 18%, respectively (Fig. 5). By contrast, high concentrations of antireceptor IgG inhibited both ^{125}I-insulin and ^{125}I-MSA binding to human fibroblasts by >95%. Approximately 5–20-fold higher concentrations of IgG are required to produce inhibition of ^{125}I-MSA binding equivalent to that of ^{125}I-insulin [12, 64]. It is not known whether the same molecules in a heterogeneous population of antibodies inhibit binding to both receptors. If this were the case, these results would suggest that the insulin-sensitive MSA receptor of human fibroblasts shares antigenic determinants with insulin receptors in a species-specific and possibly tissue-specific fashion.

There is little evidence to suggest that ^{125}I-MSA is internalized by chick embryo fibroblasts. By contrast, after EGF [55] and LDL [69] bind to surface receptors in human fibroblasts at higher temperatures (37°C, but not 4°C), they are taken into the cell in endocytotic vesicles and degraded in lysosomes. When ^{125}I-MSA binds to chick embryo fibro-

*Megyesi et al initially reported inhibition of binding of ^{125}I-labeled NSILA-s (≅15% pure, containing IGF-I and IGF-II) to rat liver plasma membranes by insulin and proinsulin [63]. Subsequently, they presented more complete dose-response curves showing no inhibition of ^{125}I-NSILA-s (or ^{125}I-MSA) binding to the same liver membrane preparations by insulin at high concentrations [62]. We have not observed inhibition of binding of ^{125}I-MSA, ^{125}I-IGF-I, or ^{125}I-IGF-II binding to the same Neville liver membrane preparations by insulin or proinsulin [2, 11, 24, 42].

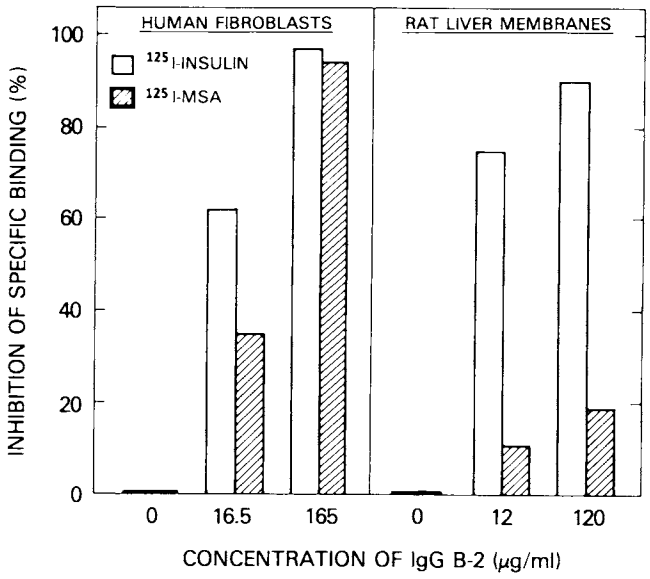

Fig. 5. Inhibition of binding to insulin receptors and MSA receptors by anti-receptor IgG B-2. IgG from patient B-2 [65, 66] with circulating antibodies to insulin receptor was purified by precipitation with 33% ammonium sulfate and DEAE-cellulose chromatography as previously described [67]. Percent inhibition of specific binding of ^{125}I-insulin (open bar) or ^{125}I-MSA (hatched bar) to human fibroblast cultures (left panel), or rat liver plasma membranes (right panel) by the indicated concentration of IgG B-2 is shown.

Binding assays were performed as previously described. Incubation conditions were as follows: a) human fibroblasts, ^{125}I-insulin, incubated 2 h at 15°C[68]; b) human fibroblasts, ^{125}I-MSA, incubated 3 h at 15°C [25]; c) ^{125}I-insulin and ^{125}I-MSA, incubated with rat liver membranes for 17 h at 2°C in 0.05 M Tris-HCl, pH 7.5, containing 10 mg/ml bovine serum albumin [42, 61].

blasts under standard binding conditions (3 h, 22°C) or at 37°C for 1 h, however, there is little evidence for degradation: ^{125}I-MSA remaining in the incubation medium and cell-associated ^{125}I-MSA extracted with Triton-urea-acetic acid remain physically intact [24]. No significant differences were observed in the rate or extent of dissociation of ^{125}I-MSA bound to chick embryo fibroblasts under diverse conditions [22°C, 3 h (steady state): 22°C, 1 h; 4°C, 19 h; 37°C, 1 h] and dissociated at 37°C (faster) or 22°C (slower). These results suggest that the major fraction of ^{125}I-MSA bound to chick embryo fibroblasts is not internalized and degraded or rendered inaccessible.

The properties of MSA and insulin most highly conserved are those determining biological activity and receptor binding. Somatomedin binding proteins in rat serum do not interact with insulin [39]. Rat insulin does not react with rabbit antibodies to MSA [32]. MSA does not react in radioimmunoassay for guinea pig and porcine insulin [J. Rosenzweig, unpublished results], nor does it react with monoclonal antibodies to rat insulin [70; J. Schroer, A. B. Knight, M. M. Rechler, unpublished observations].

In summary, insulin and the IGFs have separate receptors, but they frequently interact with the receptor for the other peptide. The receptors in some cases may share structural features recognized by anti-receptor antibodies. Insulin and the IGFs also have a similar spectrum of biological activities. We shall next turn to identifying which biological function is mediated by which receptor.

Mitogenic Effects

Growth effects in fibroblasts. MSA stimulates the incorporation of [^{3}H]thymidine into DNA in chick embryo fibroblasts [42, 71], human skin fibroblasts [10, 25] and Balb c/3T3 mouse fibroblasts [71]. Chick embryo fibroblasts are most sensitive: half-maximal stimulation occurred at ≅30 ng/ml. Human fibroblasts and 3T3 cells require 10-fold higher concentrations. High concentrations of MSA (⩾ 1 μg/ml) increase DNA content and cell number in chick fibroblasts [10, 43] and 3T3 cells [71]. Cell multiplication has not been demonstrated in human fibroblasts. Even in chick embryo fibroblasts, the doubling time in medium containing MSA is only about one-third that in serum-supplemented medium [10].

These results, obtained in serum-free, hormone-free medium (Eagle's Minimum Essential Medium supplemented with bovine serum albumin), tend to indicate that MSA is a less potent mitogen in some cells than platelet-derived growth factor (PDGF) [72, 73], fibroblast growth factor (FGF) [74], and epidermal growth factor [55]. However, recent studies have indicated that plasma factors (such as somatomedins) are not themselves sufficient to induce DNA synthesis in Balb c/3T3 cells [75]. Rather, a two-stage sequence is required in which cells are first made "competent" to respond to plasma factors (by addition of PDGF or FGF), and then are induced to "progress" to S phase by plasma factors that include somatomedin C and MSA [76]. In addition, Gospodarowicz [77] has observed that a variety of cell types that grow in serum but not in plasma when plated on plastic culture dishes, grow equally well in medium containing plasma or serum when maintained on an extracellular matrix. In the light of these results, the mitogenic role of MSA and other somatomedins should be reevaluated in cells made competent by addition of PDGF or by plating on extracellular matrix. Plasma factors may indeed be important mitogens in vivo.

Hypothesis: MSA receptors mediate growth effects of insulin and MSA in fibroblasts. Radiolabeled MSA binds to a receptor in chick embryo fibroblasts with properties that led us to consider it the probable receptor mediating the growth response [2, 10, 24, 42, 43]. The relative potencies of MSA:insulin:proinsulin for inhibiting binding to this receptor and for stimulating thymidine incorporation were 100:50:20. The absolute concentrations of MSA inhibiting binding and stimulating thymidine incorporation were similar [25]. Maximally effective concentrations of MSA, insulin, and proinsulin did not stimulate thymidine incorporation additively [43]. The human insulin-like growth factors also stimulate DNA synthesis in chick embryo fibroblasts: IGF-I and IGF-II were active at 10-fold lower concentrations than MSA [35, 45, 78]; somatomedin A [10] was equipotent with MSA. The human IGFs appear to interact with the receptor identified by ^{125}I-MSA binding, with relative potencies appropriate to their biological potency [10, 11]. Together, these results suggested that the MSA receptor in chick embryo fibroblasts might mediate the mitogenic effectors of MSA, the human IGFs, insulin, and proinsulin.

Human fibroblasts possess an MSA receptor with the same relative affinities for MSA, insulin, and proinsulin as the chick fibroblast MSA receptor [25] (Fig. 3). These peptides stimulate thymidine incorporation into DNA [25, 79, 80] (Fig. 6). The shape of the dose-response curve with insulin was less steep than the curve obtained with MSA for unexplained reasons, making it difficult to determine relative potencies. Proinsulin was ≅50% as potent as insulin and gave a parallel dose-response curve to insulin [81]. Although the process is probably less straightforward than in chick fibroblasts, we have pro-

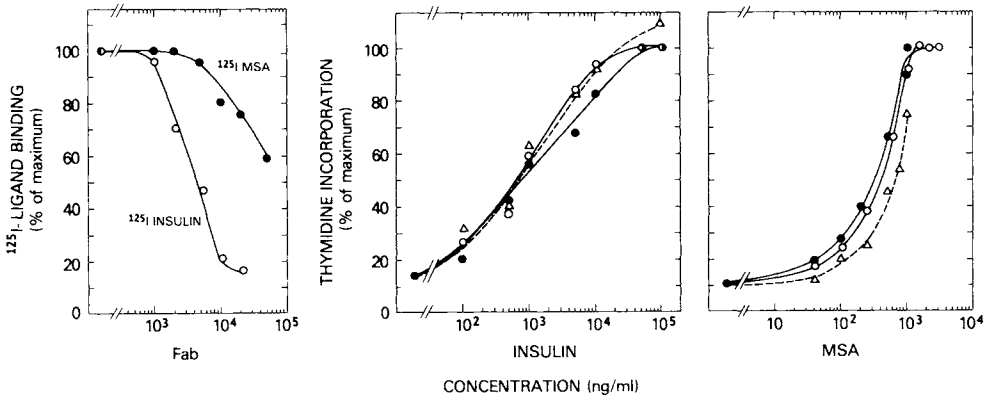

Fig. 6. Left: inhibition of ^{125}I-labeled porcine insulin and ^{125}I-labeled MSA II-1 binding to human fibroblast cultures by Fab fragments of antireceptor IgG. The Fab fragments were prepared from the serum of patient B-2 with the syndrome of extreme insulin resistance and acanthosis nigricans [65, 66]. The IgG fraction, containing antibodies to the insulin receptor, was purified by Protein A–Sepharose chromatography [80]. The IgG was digested with papain and chromatographed on Protein A–Sepharose. Undigested IgG and Fc fragments bind to Protein A; Fab fragments do not [80].

^{125}I-insulin (○) or ^{125}I-MSA (●) and the indicated concentrations of Fab fragments were incubated for 2 h at 15°C with 5 × 10^6 fibroblasts in 0.5 ml of Hepes binding buffer, pH 8.0 [25, 80]. Cell-associated radioactivity was determined following microfuge centrifugation as previously described [25].

Inhibition of ^{125}I-MSA binding to human fibroblasts by DEAE-purified IgG from patient B-2 has previously been observed [64]. Interestingly, other MSA receptors (eg, chick embryo fibroblasts, rat liver membranes) were not inhibited by the same IgG preparations [M. M. Rechler, J. M. Podskalny, C. R. Kahn, S. P. Nissley, manuscript in preparation]. Redrawn from [80] with permission.

Right and center: effect of antireceptor Fab fragments on insulin-stimulated (center) and MSA-stimulated (right) [^{3}H]thymidine incorporation in human fibroblasts. Confluent fibroblast cultures in serum-free medium were incubated with the indicated concentrations of insulin (center) or Sephadex G-75 MSA II (right) in the presence of 0 (●), 4 μg/ml (○), or 10 μg/ml (△) of Fab. After 18 h incubation, the cultures were pulsed with [^{3}H]thymidine for 30 min, and incorporation of radioactivity into acid-precipitable DNA was determined as previously described [79]. Neither antireceptor Fab fragment nor antireceptor IgG stimulated thymidine incorporation when added separately [80]. Redrawn from [80] with permission.

posed that the mitogenic effects of insulin, MSA, and human insulin-like growth factors in human fibroblasts are mediated by MSA/IGF receptors rather than by insulin receptors [2, 25, 79].*

Demonstration that the growth effects of insulin and MSA in fibroblasts are not mediated by the insulin receptor. Fab fragments prepared from IgG from the serum of a patient with insulin resistance resulting from circulating antibodies to insulin receptors are potential antagonists of insulin receptors and insulin receptor-mediated biological effects [80]. Dose-dependent inhibition of ^{125}I-insulin binding to insulin receptors on human fibroblasts by increasing concentrations of antireceptor Fab fragments is observed (Fig. 6). Blockade of MSA receptors required higher concentrations of Fab (Fig. 6), making it possible to select Fab concentrations (4 and 10 μg/ml) at which insulin binding to insulin receptors

*Additional support for this hypothesis has come from the observation that insulin isolated from casiragua, a hystricomorph [82], is 20-fold more potent in stimulating thymidine incorporation in human fibroblasts than glucose oxidation in adipocytes [81].

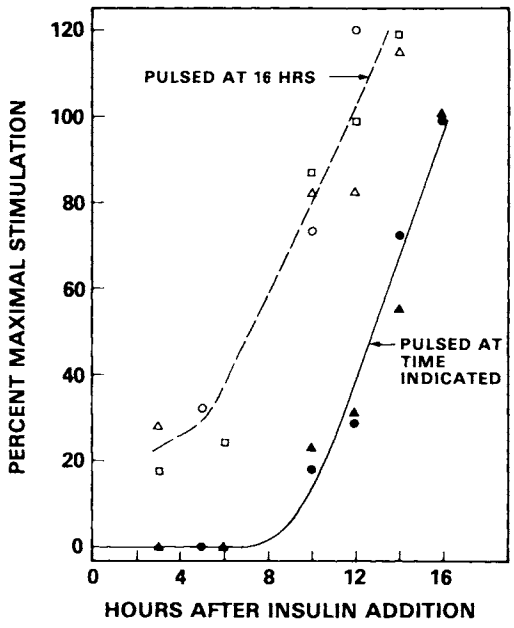

Fig 7. Incorporation of [^{3}H]thymidine into the DNA of human fibroblasts following incubation with insulin for different durations Confluent, serum-starved, quiescent fibroblast cultures received 20 μg/ml of porcine insulin at time zero. Some cultures were pulsed with [^{3}H]thymidine for 30 min beginning at the times (3–16 h) after insulin addition indicated (●, ▲) Parallel cultures (○, △, □) received insulin (20 μg/ml) for the indicated times (3–13 h), following which fresh serum-free medium (without insulin) was added The incubations were continued until 16 h after insulin addition, at which time these cultures were pulsed for 30 min with [^{3}H]thymidine as previously described [79].

was effectively blocked, but MSA receptors were negligibly affected. The ability of insulin and MSA to stimulate thymidine incorporation in human fibroblasts was examined in the presence and absence of these concentrations of Fab (Fig. 6). No significant inhibition (reflected in a rightward shift of the dose-response curve or decreased amplitude of the response) was observed.

Provided that insulin receptors were blocked by anti-receptor Fab fragments under conditions of the thymidine incorporation experiment and for the time required for insulin to exert its mitogenic effects, these results would suggest that blockade of insulin receptors has no effect on insulin- and MSA-stimulated thymidine incorporation; that is, these effects are not mediated by insulin receptors. As seen in Figure 7, insulin must be continuously present for 12–14 h for maximal stimulation of thymidine incorporation to occur. That is, human fibroblasts are not committed to DNA synthesis until approximately 4 h before the start of DNA synthesis. Similar results have been reported in chick embryo fibroblasts [83]. Since low concentrations of ^{125}I-insulin tracer (<1 ng/ml) are degraded by fibroblasts during incubation at 37°C in culture media, it is difficult to verify directly that antireceptor Fab fragments continually block insulin binding under the prolonged incubation conditions of the thymidine incorporation assay required for commitment to DNA synthesis. Independent evidence suggests that this may be the case. Antireceptor IgG inhibits insulin binding but retains some insulin-like biological activity. Under thymidine incorporation bioassay conditions, antireceptor IgG produced a small but sig-

nificant increase in the protein content of the fibroblast cultures, similar in magnitude to that produced by incubation with insulin or MSA [80]. Thus, it seems likely that insulin receptors were blocked under the experimental conditions and that the growth effects of insulin and MSA in human fibroblasts are mediated by receptors other than insulin receptors, possibly MSA/IGF receptors.

It has not been possible to perform the analogous experiments in chick embryo fibroblasts. Antireceptor Fab fragments did not inhibit MSA binding to the MSA receptor of chick fibroblasts [G. L. King, unpublished results]. However, insulin binds poorly to chick embryo fibroblasts [24], and these insulin receptors are incompletely inhibited by antireceptor Fab fragments (50–60% inhibition by 25 μg/ml Fab [G. L. King, unpublished results]) and antireceptor IgG (50% inhibition by 400 μg/ml IgG [M. M. Rechler, unpublished results]).

Mitogenic effects in other cell types. Two recent studies suggest that our hypothesis that insulin acts via IGF receptors to stimulate growth, originally formulated for cultured fibroblasts, may not hold for all cultured cells. Koontz [84] described the stimulation of thymidine incorporation in H4 cells derived from a minimal deviation Reuber hepatoma by low concentrations of insulin; half-maximal stimulation occurred at 50–100 pM. The sensitive dose-response curve for insulin suggested an insulin receptor-mediated response. Proinsulin was ≅1% as effective as insulin, consistent with this interpretation. Anti-insulin receptor IgG prepared from the serum of a patient described by Baldwin et al [85] inhibited binding of radiolabeled insulin to the insulin receptors of H4 cells. Although anti-receptor IgG B-2 did not stimulate thymidine incorporation in fibroblasts [80], the antireceptor IgG used by Koontz stimulated thymidine incorporation in H4 cells. Together, these results suggest that insulin receptors may mediate growth effects in hepatoma cells.

The different effects of anti-insulin receptor IgG on thymidine incorporation – stimulation in H4 cells, no stimulation in fibroblasts – may result from the fact that the IgG preparations were from different patients or that different target cells were used. For example, Kahn et al [86] reported that serum from 3 patients with antibodies to insulin receptors either inhibited (1 patient) or stimulated (2 patients, different dose-response curves) glucose oxidation in rat adipocytes. Of several liver-derived membranes or cells that we have examined for MSA binding, only one (BRL 61t) has an MSA receptor that is inhibited by insulin [33; M.M. Rechler, unpublished results]. Insulin does not cross-react (at 10^3 molar excess) with the MSA receptor of the Neville preparation of liver plasma membranes [10, 24] from Sprague-Dawley rats [2, 10, 24, 42],* HTC hepatoma cells [87], BRL 3A2 or BRL 3A cells [2, 10, 24, 33; M. M. Rechler and J. M. Podskalny, unpublished observations]. This suggests that if insulin acts as a mitogen in liver, it must do so via its own receptor.

A second system in which insulin appears to act as a mitogen via the insulin receptor occurs in a series of variant cell lines of Cloudman S91 melanoma cells [88]. Insulin at low concentrations inhibits the growth of S91 cells. Variant cell lines resistant to this inhibitory effect were selected. One of these lines was, in fact, dependent upon insulin for growth. Insulin stimulated cell multiplication in the dependent line at low concentrations (ED_{50} 0.1 nM); MSA was approximately 0.2% as potent. These results were similar to the relative potencies of insulin and MSA as inhibitors of ^{125}I-insulin binding to the insulin receptors of the insulin-dependent melanoma line: K_D 1–2 nM, MSA ≅1% as potent as insulin. The simplest interpretation of these results is that insulin utilizes insulin receptors to stimulate the growth of the insulin-dependent melanoma line.

*See footnote on page 13.

By contrast, Florini et al [89] have presented evidence that MSA is a mitogen for rat myoblasts in culture and presumably utilizes MSA/IGF receptors. Multiplication was stimulated significantly by MSA at 10^{-7} M, but not by insulin at 10^{-6} M.

Sato and his colleagues have established hormonal supplements of defined culture medium that allow a variety of cell types to replicate in the absence of serum [90]. One component required for growth of all cells tested is insulin, typically added at high concentrations (2–5 μg/ml). At these concentrations, insulin might be acting via an MSA/IGF receptor rather than via an insulin receptor. It would be of interest to evaluate whether MSA can substitute for insulin in some of these cell types and to determine the reactivity of their MSA receptors with insulin.

Glucose Oxidation: Adipocytes

The original name for the insulin-like growth factors, nonsuppressible insulin-like activity, arose from the observation that human plasma contained insulin-like biological activity in an in vitro bioassay in fat that could not be neutralized (suppressed) by antibodies to insulin [8]. Partially purified NSILA also was active in vivo, inducing hypoglycemia in adrenalectomized rats [91]. Purified IGF-I, IGF-II, and MSA have insulin-like activity in adipose tissue in vitro; for example, stimulation of glucose oxidation and lipogenesis [35, 36]. The purified insulin-like growth factors compete for ^{125}I-insulin binding to insulin receptors of adipocytes and other tissues (Fig. 8) [2, 24]. In addition, radioiodine-labeled IGF-I, IGF-II and MSA also bind to IGF receptors in adipocytes [92]. This is illustrated using ^{125}I-MSA (Fig. 8). Insulin did not inhibit ^{125}I-MSA binding to adiocytes but instead increased binding approximately 50% in a dose-dependent fashion (Fig. 8). Similar enhancement of ^{125}I-labeled NSILA [92] and IGF-II [9, 78] binding to adipocytes and ^{125}I-MSA binding to rat myoblasts [J. Florini, personal communication] by insulin have been observed. King et al [93] have presented evidence that the enhancement of ^{125}I-MSA binding to the MSA receptor by insulin requires participation of the insulin receptor and has a concentration dependence similar to insulin stimulation of glucose oxidation.

As in human fibroblasts, Fab fragments of antireceptor IgG offer a tool with which to inhibit adipocyte insulin receptors selectively, without significantly affecting MSA receptors. Antireceptor Fab fragments at 1, 3, and 5 μg/ml inhibited ^{125}I-insulin binding to adipocytes by 10%, 25%, and 80%, respectively; ^{125}I-MSA binding was not significantly decreased (Fig. 9).

Although antireceptor IgG from NIH patient B-2 stimulates glucose oxidation, the Fab fragments prepared from this IgG have no stimulatory activity and are pure antagonists of insulin action [80, 94] (Fig. 9). Increasing concentrations of Fab progressively shifted the dose-response curve for insulin stimulation of glucose oxidation to the right. This is the result expected if the Fab fragments were competitive inhibitors of insulin action. (The alternative possibility that the Fab fragments were noncompetitive inhibitors cannot rigorously be excluded at the concentrations of Fab that it was practicable to test.) Assuming that only 10–20% of insulin receptors need be occupied to produce a maximal response in adipocytes ("spare receptors"), either a competitive inhibitor or a non-competitive inhibitor at low concentration would shift the dose response curve to the right. At higher concentrations, however, a noncompetitive inhibitor but not a competitive inhibitor also would decrease the magnitude of the response.

The effect of antireceptor Fab fragment on MSA-stimulated glucose oxidation was next examined. If MSA acted through the IGF/MSA receptor, which was not blocked by antireceptor Fab, the dose-response curve would be unchanged by the presence of anti-

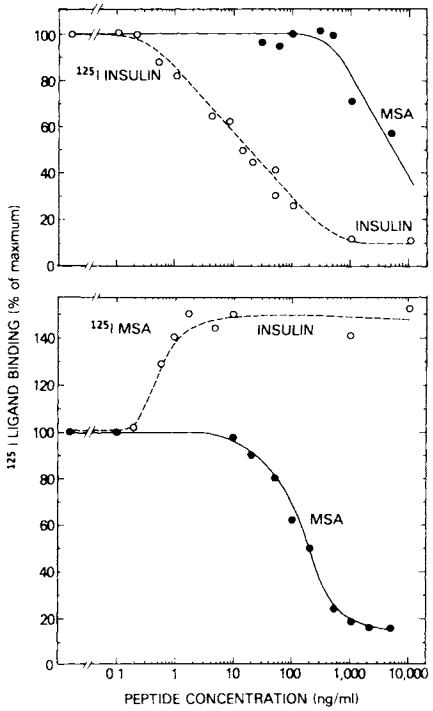

Fig. 8. Binding of ^{125}I-insulin (top) and ^{125}I-MSA (bottom) to isolated rat adipocytes. Adipocytes were prepared from epididymal fat pads of 100–160 gm Sprague-Dawley rats by collagenase treatment [80]. Adipocytes (1.2×10^5 cells), ^{125}I-ligand, and unlabeled insulin (○) or MSA (Sephadex G-75 peak II) (●) at the indicated concentrations were incubated in 0.5 ml pH 7.4 Krebs-Ringer buffer containing 20 mg/ml bovine albumin. Steady-state conditions were used: for ^{125}I-insulin, 20 min at 37°C; for ^{125}I-MSA, 40 min at 24°C. Adipocyte-associated radioactivity was recovered in the layer above dinonylphthalate following microfuge centrifugation. Percent of maximum binding is plotted; maximum binding represents 4% of input tracer radioactivity for both experiments. Redrawn from [80] with permission.

receptor Fab. If, however, MSA acted through the insulin receptor to stimulate glucose oxidation, blockade of the insulin receptor by Fab fragments should shift the dose-response curve to the right, as observed with insulin. As seen in Figure 9 (right panel), the latter result was obtained, indicating that some if not all of MSA's activity in adipocytes occurs via the insulin receptor. It should be noted that the effects of antireceptor Fab are specific for agents that stimulate glucose oxidation via the insulin receptor; stimulation by vitamin K_5 and spermine, which act by different mechanisms, is not inhibited [80]. The function of the MSA/IGF receptor in adipocytes, as well as the significance of the interaction of this receptor with insulin and/or the insulin receptor, remains to be elucidated.

Glucose Incorporation and Amino Acid Transport in Human Fibroblasts

The preceding sections have focused on two extremes of insulin action: an acute metabolic effect in a traditional target tissue (glucose oxidation in adipocytes) and a chronic growth effect (DNA synthesis in cultured fibroblasts). We now examine two

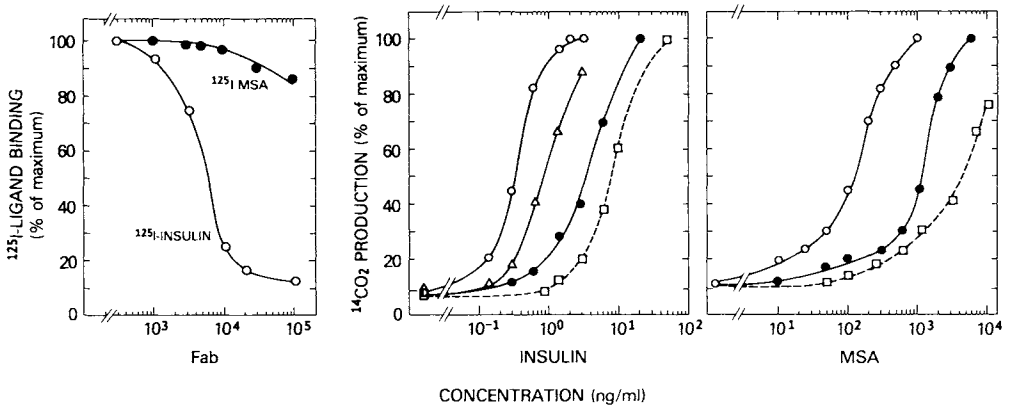

Fig. 9. Left: inhibition of ^{125}I-insulin and ^{125}I-MSA binding to isolated rat adipocytes by Fab fragments of antireceptor IgG. Fab fragments were prepared as described in Figure 6. Preparation of adipocytes and binding conditions were as described in Figure 8. Percent of maximum binding is plotted for different Fab concentrations.

Right and center: effect of antireceptor Fab fragments on insulin-stimulated (center) and MSA-stimulated (right) glucose oxidation in rat adipocytes. Glucose oxidation was quantitated by the conversion of (U)^{14}C-glucose to $^{14}CO_2$ during 1 h of incubation as previously described [92]. Insulin (center) and MSA (right) were added at the indicated concentrations in the presence of 0 (○), 3 μg/ml (△), 5 μg/ml (●), or 10 μg/ml (□) of antireceptor Fab fragment. Redrawn from [80] with permission.

acute metabolic effects in human fibroblasts: transport of the non-metabolizable amino acid, α-aminoisobutyric acid (AIB) and glucose uptake. Our interest in cultured fibroblasts derives from the fact that they represent one of a limited number of cell types that can be isolated from patients with disorders of target-organ resistance to insulin or IGFs and propagated for 50–60 generations in culture, thereby allowing expression of their genotype free from influences of the physiological environment at the time the initial cells were removed by biopsy from the patient. Human fibroblasts possess both classic insulin receptors [68] and an MSA/IGF receptor that interacts with insulin [25] (Fig. 3). We recently have reported a profound reduction of insulin binding to insulin receptors on fibroblasts cultured from an infant with leprechaunism, severe insulin resistance, and intrauterine growth retardation [95]. Prior to evaluating the functional consequences of this defective insulin binding, we have attempted first to define the biological functions mediated by insulin in fibroblasts and to determine whether insulin produces these responses via an insulin receptor or an MSA/IGF receptor.

Glucose incorporation. Howard et al [96] have demonstrated that insulin at low concentrations stimulated total glucose incorporation into human fibroblast cultures. Stimulation is seen after 30 min of exposure to insulin; maximal stimulation requires 2 h of incubation. Fibroblasts incubated with [^{14}C]glucose for 20 min show increased incorporation in all compartments in insulin-treated cells: glycogen, acid-soluble nucleotides, lactic acid, etc. Using the procedures described by Howard et al [96] with some modifications, we have confirmed that insulin induces a 60–100% increase in [^{14}C]glucose incorporation. Half-maximal stimulation was observed with ≈ 5 ng/ml of insulin [97]. Proinsulin stimu-

lated incorporation to the same extent, but required 50-fold higher concentrations (Table VI) [97]. These results suggested that insulin and proinsulin probably were acting via the insulin receptor.

MSA also was a potent stimulator of glucose incorporation in human fibroblasts (Table VI). Surprisingly, and in contrast to its relative impotence in adipocytes, MSA was 12% as potent as insulin; that is, it was 6 times more potent than proinsulin. Since MSA interacts more weakly with the human fibroblast insulin receptor than proinsulin (<1% as potent as insulin compared to 5%) [24, 68], this result provisionally suggests that the stimulation of glucose incorporation by MSA cannot be explained completely by its interaction with insulin receptors, and it probably represents an activity mediated by the MSA/IGF receptor.

Similar reasoning led Meuli and his colleagues to conclude that insulin and NSILA-s elicited the same biological effects (3-O-methyl-glucose transport, glucose uptake, lactate production) in the perfused rat heart by interacting with different receptors [98, 99]. The preparation of NSILA-s used was 1/60th as potent as insulin in adipocytes, but 1/2–1/5th as potent as insulin in rat heart muscle.

Similar results have been reported recently by Poggi et al in mouse soleus muscle [100]. IGF-I stimulated glucose metabolism (2-deoxyglucose uptake, glycolysis, glycogen synthesis) 4–9% as potently as insulin. Since insulin did not bind to the IGF-I receptor in this tissue, these results were interpreted as indicating that insulin acts via the insulin receptor and that IGF-I acts at least in part via the IGF receptor [100].

Amino acid transport. Neutral α-amino acids may be transported by several specific transport systems in animal tissues [101]. Although the non-metabolizable amino acid, AIB, may be transported by 3 different systems [101], stimulation of AIB transport in hepatocytes by insulin appears to involve selective stimulation of the A-transport system [102]. Of the 3 systems that potentially transport AIB, only the Na^+-dependent A-system is inhibited by N-methyl-AIB.

The ability of insulin, proinsulin, and MSA to stimulate uptake of [^{14}C]methyl-AIB via the A-transport system has been examined in human fibroblasts [97] (Table VI). Experimental procedures are similar to those described by Hollenberg [103, 104]. Insulin stimulated methyl-AIB uptake approximately 2-fold [97]. Half-maximal stimulation occurred at approximately 5 ng/ml [97], similar to values previously reported by others [104, 105]. Proinsulin was approximately 5% as potent as insulin [97] (Table VI), consistent with published results [104] and with the potency of proinsulin as an inhibitor of ^{125}I-insulin binding to insulin receptors [68].

As seen for glucose incorporation in human fibroblasts, MSA was considerably more potent in stimulating methyl-AIB transport (~50% as potent as insulin) than would have been anticipated from its interaction with insulin receptors [97] (Table VI). Presumably it acts through the MSA/IGF receptor. In preliminary experiments, IGF-I also stimulated both methyl-AIB uptake and glucose incorporation with a potency greater than would be expected if it acted predominantly via the insulin receptor [M. M. Rechler, A. B. Knight, unpublished results]. Hollenberg and Fryklund [106] previously reported that somatomedin A stimulated AIB transport in human fibroblasts with a high potency, suggesting that it acted via a somatomedin receptor.

As summarized in Table VII, insulin and MSA are capable of stimulating AIB transport in different experimental systems by different mechanisms. In human fibroblasts and HTC hepatoma cells, insulin and proinsulin appear to utilize insulin receptors, whereas MSA and other IGFs appear to utilize the MSA/IGF receptor (Table VII). By contrast, in

TABLE VI. Relative Potencies of Insulin, MSA, and Proinsulin for Different Biological Responses and Receptors

	Insulin[a]	Proinsulin[a]	MSA[a]
1) Insulin receptors: adipocytes [80], human fibroblasts [68], IM-9 lymphoblasts [24], rat liver membranes [54][b]	100	5	0.1–1.0
2) MSA receptors: chick fibroblasts, human fibroblasts [24, 25, 43]	100	40	200
3) Adipocytes: glucose oxidation [80]	100	5	0.2
4) Chick fibroblasts: thymidine incorporation[c]	100	40	200
5) Human fibroblasts			
Glucose incorporation [97][d]	100	2	12
Methyl AIB transport [97][e]	100	5	50
6) Hepatoma (HTC) cells: Tyrosine aminotransferase induction [87][f]	100	13	28
7) Perfused rat heart: glucose transport and metabolism [98, 99]	100	NT[g]	20–40[h]
8) Muscle, mouse soleus: glucose uptake and metabolism [100]	100	NT	4–9[i]
9) Chondrocytes (Swarm rat chondrosarcoma): proteoglycan synthesis [100a]	100	3	1

[a]Porcine insulin, porcine proinsulin, Sephadex G-75 peak II MSA.
[b]M. M. Rechler, unpublished results.
[c]Proinsulin also is 40% as potent as insulin in stimulating thymidine incorporation in human fibroblasts [81]. As discussed in the text, the dose-response curve for MSA is nonparallel (steeper), so that relative potency is difficult to assess.
[d]A. B. Knight, unpublished results.
[e]M. M. Rechler, unpublished results.
[f]Relative potencies based on 4 paired experiments each for insulin-MSA and insulin-proinsulin.
[g]NT = not tested.
[h]Performed with partially purified NSILA-s, 1/60th as potent as insulin in adipocytes.
[i]Performed with IGF-I.

chick embryo heart, MSA, insulin, and proinsulin all appear to stimulate AIB transport via the MSA receptor and not the insulin receptor [107]. (It should be emphasized that this mechanism requires that the MSA/IGF receptor shows substantial cross-reactivity with insulin and proinsulin.) This summary clearly illustrates the necessity of determining the relative biological potencies of both classes of peptides, as well as defining the properties of the receptors with which these peptides interact, before reaching conclusions about the receptor that mediates a given biological response.

HTC Rat Hepatoma Cells: Induction of Tyrosine Aminotransferase (TAT)

Insulin causes a 2-fold increase in the amount of TAT in HTC cells in which TAT has been induced by dexamethasone, by decreasing the rate of TAT degradation [112, 113]. Half-maximal stimulation is achieved with ≅30 ng/ml of insulin [87]. Proinsulin and MSA are 9% and 28% as potent as insulin, respectively [87]. HTC cells possess both an insulin receptor and an MSA receptor [87]. ^{125}I-insulin binding is inhibited 50% by ≅33 ng/ml of insulin [87]. Proinsulin inhibits insulin binding with a potency slightly lower than its biological potency in HTC cells [87]. The MSA receptor of HTC cells is relatively insensitive (50% inhibition of ^{125}I-MSA binding by 200 ng/ml unlabeled MSA) and does not interact with insulin (no inhibition of ^{125}I-MSA binding by 10 μg/ml insulin [87]. These results

TABLE VII. Predominant Receptors Mediating the Stimulation of AIB Transport by Insulin and Insulin-Like Growth Factors in Different Experimental Systems

Experimental system	Peptide	Receptor used	Reference
1) Human fibroblasts	Insulin	Insulin	[97, 104, 105][a]
	Proinsulin	Insulin	[97, 104][a]
	MSA	MSA/IGF	[97][a]
	IGF-I	MSA/IGF	[97][a]
	Somatomedin A	MSA/IGF	[106]
2) Chick embryo fibroblasts	MSA	MSA/IGF (?)[b]	[83]
3) Chick embryonic heart	MSA	MSA/IGF[c]	[107]
	Insulin	MSA/IGF[c]	[107]
	Proinsulin	MSA/IGF[c]	[107]
4) Rat thymocytes	Insulin	Insulin[d]	[108]
5) HTC cells	Insulin	Insulin	[87]
	Proinsulin	Insulin	[87]
	MSA	MSA[e]	[87]
6) Rat myoblasts	MSA	MSA/IGF (?)[f]	[109]
	Insulin	(?)[f]	[109]
	Somatomedin A	(?)[f]	[110]
7) Rat hepatocytes	Insulin	Insulin (?)[g]	[102, 111]

[a]M.M. Rechler, unpublished results [107a].

[b]Insulin not tested.

[c]The relative potencies for stimulation of [^{14}C]methyl-AIB transport and inhibition of ^{125}I-MSA binding were identical: MSA:insulin:proinsulin = 2–3:1:0.2–0.3.

[d]The dose-response curve for insulin stimulation of AIB transport was complex. Assignment was based on the fact that one K_D for binding and biological response were similar. Studies with insulin analogues or insulin-like growth factors were not performed.

[e]The MSA receptor of HTC cells does not interact with insulin [87].

[f]MSA and insulin stimulation of AIB transport in L6 rat myoblasts required high concentrations ($ED_{50} \cong 1 \mu g/ml$; maximal response at $\cong 10 \mu g/ml$) [109]. Concentration dependence was similar for the two peptides. Stimulation by somatomedin A [110] was observed at high concentration (1 μg/ml); a dose-response curve was not presented.

[g]ED_{50} for insulin 12–18 ng/ml [102], 40 ng/ml [111]. Insulin-like growth factors not tested.

suggest that insulin and proinsulin act predominantly by an insulin receptor-mediated mechanism. The greater relative potency of MSA suggests that it exerts at least part of its biological effect via the MSA receptor.

Proteoglycan Synthesis: Chondrocytes

The existence of growth hormone-dependent substances in rat serum that presumably mediate the action of growth hormone on cartilage was first appreciated using as in vitro bioassay the stimulation of [^{35}S]sulfate incorporation into glycosaminoglycans in costal cartilage from hypophysectomized (hypox) rats [114]. Sulfate incorporation assays in hypox rat costal cartilage or chick pelvic leaflets guided the purification of somatomedin C [7] and somatomedin A [6] from human serum. Moreover, cartilage assays on serum samples have been the classic biological assay for somatomedin activity in different physiological and pathological states [115]. Over the years, it has been appreciated that supraphysiological concentrations of insulin are capable of mimicking the actions of somatomedins in cartilage [116, 117], presumably by weak cross-reaction with somatomedin receptors [57]. The studies to be described present evidence for an alternative mechanism; namely, that

insulin and MSA stimulate proteoglycan synthesis in chondrocytes prepared from the Swarm rat chondrosarcoma by an insulin receptor-mediated mechanism [100a].

Proteoglycan synthesis in rat chondrosarcoma chondrocytes has been extensively studied by Hascall and co-workers [118]. Insulin and MSA stimulated proteoglycan synthesis 2–3-fold; the proteoglycan was normal in size, chondroitin sulfate content, and ability to aggregate [119]. Half-maximal response was observed with 1 ng/ml of insulin, 30 ng/ml of proinsulin, and 100 ng/ml of MSA [100a, 119]. Chondrocytes were shown to possess a typical insulin receptor [100a] and an MSA receptor that did not react with insulin at high concentrations [100a]. These results strongly suggest that insulin stimulates proteoglycan synthesis through an insulin receptor; MSA could act through the insulin receptor and/or the MSA receptor.

Miscellaneous Biological Actions of MSA

Casein synthesis: mouse mammary epithelium in culture. Insulin at high concentrations acts synergistically with glucocorticoids and prolactin to stimulate synthesis of milk proteins by mouse mammary explants [120]. Casein synthesis by explants from midpregnant mice was evaluated by immunoprecipitation after 48 h of incubation with [^{3}H]amino acids, as previously described [121]. In the presence of hydrocortisone (1 μg/ml) and prolactin (5 μg/ml), insulin (5 μg/ml) and MSA (5 μg/ml) stimulated casein synthesis, whereas no stimulation was observed with MSA at 1 μg/ml [T. Oka, C. Hori, S. P. Nissley, M. M. Rechler, unpublished results]. Synthesis with 5 μg/ml MSA was ≅40% of that obtained with insulin at the same concentration. In other experiments, significant stimulation has been observed with 50 ng/ml of insulin [T. Oka, unpublished results]. These results suggest that MSA is 1–5% as potent as insulin in stimulating casein synthesis, a finding consistent with both peptides acting via the insulin receptor.

Intracellular cyclic AMP: chick embryo fibroblasts. MSA induced a rapid, concentration-dependent inhibition of PGE_1-stimulated cAMP accumulation in chick embryo fibroblasts [122]. Maximal inhibition was achieved with 100 ng/ml. Inhibition of PGE_1-stimulated and fluoride-stimulated adenylate cyclase was observed in membranes prepared from these cells [122].

Promote meiosis: Xenopus oocytes. Insulin can induce meiotic division in Xenopus laevis oocytes at high concentrations (ED_{50} 2 μM) [123]. In prelimary experiments, MSA also is a meiosis-promoting agent of stage 5 Xenopus oocytes in vitro [M. El-Etr and E. E. Baulieu, personal communication]. MSA is effective at lower concentrations than insulin (≅10-fold). Evaluation of insulin and MSA receptors is in progress.

Ornithine decarboxylase: porcine granulosa cells. MSA, like a variety of other hormones, including LH, FSH, EGF, cAMP analogs, and prostaglandins of the E series, stimulates ornithine decarboxylase activity in isolated porcine granulosa cells maintained under defined conditions in vitro [124]. The dose-response curve is quite sensitive, with ED_{50} ≈10 ng/ml MSA [124]. MSA has previously been shown to stimulate ornithine decarboxylase in 3T3 mouse fibroblasts [71].

ROLE OF INSULIN AND INSULIN-LIKE GROWTH FACTORS IN FETAL GROWTH

The hormonal determinants of intrauterine growth are poorly understood. There is little to indicate a significant role for pituitary growth hormone, since fetuses lacking fetal growth hormone or maternal growth hormone are normal in size [125]. Insulin has been

implicated by association in the pathogenesis of the large babies born to diabetic mothers [126]. The converse, insulin deficiency, has been reported in rare cases of pancreatic agenesis, and is associated with cessation of fetal growth in the third trimester, exceedingly small birthweight, and early neonatal death [127–129]. The insulin-like growth factors have received relatively little attention as fetal growth factors. In this section, we shall consider evidence implicating MSA (specifically among insulin-like growth factors) as a fetal growth factor in the rat, evidence implicating insulin receptors (and perhaps IGF receptors) in human intrauterine growth retardation in a patient with leprechaunism, and possible mechanisms for the large size of infants of diabetic mothers.

MSA as Fetal Growth Factor in the Rat

In 1979 we reported that explants of near-term fetal rat liver, placed in organ culture for up to 4 days in serum-free completely defined medium, synthesized significant quantities of peptides indistinguishable from BRL-MSA in biological, chemical, and immunological properties, and in interactions with receptors and binding proteins [36]. This observation was particularly intriguing because the liver explant system is known to faithfully recreate the developmental state, pattern of enzyme regulation, and pattern of fetal protein synthesis appropriate to the living fetus at the same gestational age [36]. This strongly suggested that fetal rat liver at near term synthesizes significant quantities of MSA-like peptides.

Moses et al [46] next examined serum from rats at different gestational ages, employing the MSA assays described in Table IV. Fetal rat serum of 16–20 days gestational age was found to contain 1–4 μg/ml of MSA by radioimmunoassay, liver membrane receptor assay, and competitive binding protein assay. Maternal serum contained 20–100 times lower amounts of MSA by immunoassay. After birth, radioimmunoassayable MSA levels began to fall to the low adult levels after 5–10 days [46].

Total somatomedin activity, measured in the chick embryo fibroblast thymidine incorporation bioassay, was similar to the level of immunoreactive MSA in fetal rat serum [46]. However, somatomedin levels determined by this bioassay were higher in maternal serum than in fetal serum, and they were substantially greater than the levels of immunoreactive MSA in maternal rat serum. These results suggest that MSA may be the predominant fetal somatomedin in the rat, but that other somatomedins, which appear after birth, represent the major postnatal species. Studies are in progress to evaluate the association of fetal somatomedin with serum binding proteins, and to determine MSA and binding protein levels in states of pathological intrauterine growth.

Leprechaunism: Genetic Disorder of Decreased Insulin Binding and Intrauterine Growth Retardation

Leprechaunism is a rare (<30 cases), heterogeneous, presumably genetic syndrome characterized by low birthweight, absent subcutaneous fat, poorly developed muscles, unusual facies, hirsutism, and frequently by hyperinsulinemia and insulin resistance [reviewed in 130]. A small subgroup of leprechaun patients with insulin resistance has been studied more intensively. Available results suggest a variety of molecular defects, including primary (cellular) decrease in insulin receptors, secondary (humoral) decrease in insulin receptors, or post-receptor defects. In patients with a post-receptor defect, insulin binding to insulin receptors would be normal, but the biological response to insulin would be decreased. Two patients have been described with a possible defect at this level: one patient from Arkansas studied by Kobayashi et al [131] had normal insulin binding to monocytes and fibroblasts,

but abnormal glucose transport*; one patient from North Carolina had normal insulin binding, but insulin function in the patient's cells was not examined [132]. Two siblings studied in Arkansas [128, 133] exhibited abnormal insulin binding to their circulating cells (erythrocytes). Binding studies to fibroblasts cultured from these patients have not yet been reported, so it is not possible to determine whether the receptor defect is secondary to humoral factors (eg, insulin, antireceptor antibodies) or to a primary cellular defect. Finally, we have had the opportunity to study fibroblasts from a leprechaun patient seen in Winnipeg, Canada [130], which exhibit a profound selective defect of insulin binding to insulin receptors [95, 134]. Since the abnormality in this patient was expressed in fibroblasts propagated for multiple generations in culture, we consider this the first demonstration of a primary, cellular, genetic defect of insulin receptors in man. Studies defining the defect in this patient will be described below.†

Binding of ^{125}I-insulin to fibroblasts from our leprechaun patient was consistently $\leqslant$20% of binding to age- and sex-matched control fibroblasts (determined per cell or per microgram of DNA, after correction for nonspecific or nonsaturable binding) [95]. Although the experimental observation is clearcut, the validity of the conclusion critically depends on eliminating possible extraneous influences that might spuriously generate these results. These include the following:

1) Cell handling. Patient and control cultures were established in Winnipeg by identical procedures and shipped to NIH at similar low passage number at the same time. Subsequent handling of stock cultures and cultures prior to experiments were performed in parallel (ie, same media, split ratio, time). Experiments were performed with patient and control cells at the same (±2) passage number. To eliminate possible variations in binding with cell growth rate, only confluent, quiescent cultures were used. Patients and control fibroblasts grew at a similar rate [J.A. Romanus, E.E. Schilling, M.M. Rechler, unpublished observations]. Cultures were examined for Mycoplasma contamination by culture techniques [95] and found negative.

2) Binding to suspended cells or fibroblast monolayers. We have routinely suspended fibroblasts by gentle and controlled trypsinization, for greater flexibility in optimizing binding assay conditions (temperature, cell concentration) [24, 25, 68, 136]. The defect in insulin binding to patient's fibroblasts was observed in suspension or in situ.

3) Control fibroblasts were representative of fibroblasts from other normal individuals. The extent and dose response of insulin binding to the control fibroblasts obtained from Winnipeg and paired with the leprechaun patient's fibroblasts did not differ significantly from a broader experience with fibroblasts cultured from forearm skin of adult donors [136, 137] and studied by the same procedures in our laboratory. The control fibroblasts from Winnipeg were established from foreskin; the patient's fibroblasts were derived from skin of the upper arm. The site from which the skin was derived does not appear to be a major variable, since similar control results were obtained with foreskin, skin from

*Insulin binding to erythrocytes from this patient appears to be decreased [131a; M. J. Elders, personal communication], suggesting that the defect may be at the level of the insulin receptor.

†Recently, a patient cared for in Minnesota was found to have decreased insulin binding to permanent cultures of B lymphocytes [135], suggesting a cellular defect possibly similar to that of the Winnipeg patient [95].

the arm of an adult, or skin from a 3-month-old female [95].

4) Degradation of ^{125}I-insulin. Following incubation with patient's fibroblasts, ^{125}I-insulin recovered from the supernate is physically intact [95] and fully capable of binding to IM-9 lymphoblasts [J. A. Romanus, unpublished results].

5) Incubation conditions. Binding of ^{125}I-insulin to patient's fibroblasts was reduced to the same extent when binding studies were performed at different times of incubation, at different temperatures, and at different pHs [95; E. E. Schilling, unpublished results].

6) Cell size. Patient's fibroblasts appear to have slightly less surface area than control fibroblasts (Coulter channelyzer, assuming suspended cells are spheres), but the magnitude of this decrease is too small to account for the reduction of insulin binding [95]. The appearance of patient's and control fibroblasts on scanning electron microscopy is similar [B. Rentier and E. E. Schilling, unpublished observations]. A more sensitive control for cell surface area is the binding of EGF, a chemically unrelated peptide, to patient and control fibroblasts. EGF binding is complicated by the fact that EGF and its receptors are internalized by fibroblasts, taken up by lysosomes where EGF (and possibly the receptor as well) is degraded [55]. Studies were performed in the presence of 10 mM NH_4Cl to inhibit lysosomal uptake and degradation (suggested by Dr. Stanley Cohen) in order to achieve a steady state of binding, and in the absence of NH_4Cl. Under both conditions, EGF binding to fibroblasts from the leprechaun patient and control was indistinguishable [95; E.E. Schilling, unpublished results]. These results indicate that the abnormality in the leprechaun patient's fibroblasts is selective, and not a general cell-surface abnormality or simple reduction in cell surface area.

Recent studies provide further fascinating insights into the nature of the genetic abnormality in the leprechaun patient's cells, and into the coupling of insulin receptors to insulin action [97, 138].

First, binding of ^{125}I-IGF-I to the IGF-I receptor (Fig. 3) of patient and control fibroblasts was examined [138]. Binding to the leprechaun patient's fibroblasts (per cell) was decreased by 78 ± 9% compared to control fibroblasts in 5 paired experiments, a reduction comparable in magnitude to that observed for insulin receptors [97, 138]. Since insulin receptors and IGF receptors react with the same peptides, and since antibodies to insulin receptors recognize determinants on the MSA/IGF receptor of human fibroblasts, it is possible that insulin receptors and IGF receptors share a common structural domain, and that this component may be altered by mutation in our leprechaun patient's cells. Alternatively, insulin receptors and IGF receptors may be present in the plasma membrane adjacent to common membrane components (? transducers), that may be altered by the leprechaun mutation and decrease binding to both receptors. Needless to say, this dual receptor defect compels us to withhold final judgment on whether the intrauterine growth retardation in leprechaunism results from a failure of insulin action via insulin receptors or IGF receptors, or a lack of IGF effect via the IGF receptor.

Second, two acute metabolic functions of insulin and MSA have been examined in the leprechaun patient's and control fibroblasts: glucose incorporation and AIB transport [97]. As discussed above, insulin (and proinsulin) appear to stimulate both responses with similar sensitivity (≅5 ng/ml) and specificity, compatible with these effects being mediated by insulin receptors. MSA, however, appears to stimulate both effects via IGF/MSA receptors (Table VI). In the leprechaun patient's fibroblasts, stimulation of glucose incorporation by insulin and MSA is greatly impaired [97]. By contrast, stimulation of AIB trans-

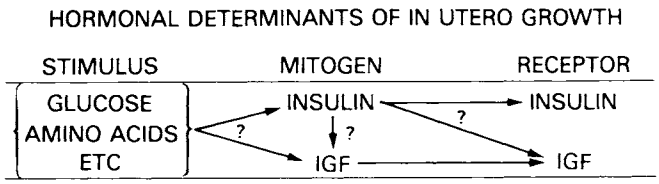

Fig. 10. Schematic representation of possible mechanisms of insulin and IGF regulation of intrauterine growth.

port by insulin and MSA is normal in magnitude in the patient's fibroblasts and slightly shifted in sensitivity [97, 107a]. One possible interpretation of these results would be the existence of functional subclasses of insulin receptors and IGF receptors or multicomponent receptors [107a]: Certain receptors (components) are coupled to effectors mediating glucose incorporation (and are abnormal in the patient's cells); other receptors (components) are coupled to effectors mediating amino acid-transport (normal in the leprechaun patient's cells). Moreover, receptors for insulin and IGF that mediate the same function (glucose incorporation or amino acid transport) appear to be coupled to the same effectors. If the coupling molecules are in proximity to the insulin and IGF binding sites, mutational alteration of those molecules coupling both binding sites to glucose uptake might be affected in our leprechaun patient's fibroblasts. These putative coupling molecules would differ from those linking insulin receptors and IGF receptors to AIB transport.

Infant of the Diabetic Mother: Pathogenesis of Large Babies

Infants of diabetic mothers have increased weight and length at birth compared to infants of normal mothers [126]. This fetal macrosomia is thought to arise by the following pathogenetic sequence: maternal hyperglycemia results in fetal hyperglycemia, which induces fetal hyperinsulinism [126]. Supporting evidence links elevated fetal insulin with large infant size [126]. Questions deserving further exploration include whether insulin acts via insulin receptors (which has been tacitly assumed), or whether the insulin-like growth factors and IGF receptors may be involved [134]. Some of the mechanistic alternatives are diagrammed in Figure 10. 1) If insulin were the responsible mitogen, it might act via insulin or IGF receptors (provided that the IGF receptors in the relevant tissues interact with insulin). 2) Alternatively, insulin might act indirectly by stimulating IGF synthesis and release. 3) IGF synthesis might increase in response to the same stimuli as those that trigger increased insulin synthesis (eg, amino acids and/or glucose). 4) IGF would act as an in utero mitogen via IGF receptors. Precedent exists for each of these mechanistic alternatives. Given the complex interrelationship of insulin and IGFs, and insulin and IGF receptors, in their biological actions and involvement in the intrauterine growth retardation of leprechaunism, we submit that these mechanisms should be considered as possible factors in the pathogenesis of the large infants of diabetic mothers.

SUMMARY AND CONCLUSIONS

1) The insulin-like growth factors (IGF-I, IGF-II, somatomedin A, somatomedin C, MSA), constitute a closely related group of polypeptides with similar chemical structure, biological activities, target organs, and recognition by antibodies, carrier proteins, and re-

ceptors. By contrast, the insulin-related growth factors (NGF, relaxin) have a more distant structural resemblance and differ in site of synthesis, biological responses, target tissues, and recognition by receptors.

2) Properties of one of the insulin-like growth factors, MSA, are described in detail. Two MSA species have been obtained in homogeneous form. The relationship among MSA species is inferred from chemical, physical, and immunological studies.

3) Comparative studies have previously established the close resemblance of MSA, synthesized by a cultured rat liver cell line to human somatomedin A, IGF-I, and IGF-II. Studies are presented that indicate the similarity between MSA and human somatomedin C in receptor and immunoassays.

A preparation of rat somatomedin partially purified from rat serum cross-reacts in some, but not all, assays for MSA, indicating that these polypeptides are not identical.

4) Most tissues possess separate receptors for insulin and for the insulin-like growth factors. Cross-reaction of insulin with IGF/MSA receptors, and of IGFs with insulin receptors is frequently observed. Because of the overlapping specificity, insulin and the IGFs frequently exhibit the same biological effects. Comparison of the relative potencies of IGF and insulin in a given bioassay with the properties of IGF and insulin receptors in that tissue provides preliminary clues as to which receptor mediates the activity of a given peptide. More direct analysis has been possible in some instances by using selective blockade of insulin receptors (and insulin receptor-mediated function) with Fab fragments of human antibodies to insulin receptors. Results of these studies include the following a) The metabolic effects of IGFs and insulin in adipocytes are mediated by insulin receptors; b) stimulation of amino acid transport may occur via both receptors (eg, HTC cells, human fibroblasts), or insulin and IGF (MSA) both may utilize the MSA/IGF receptor (embryonic chick heart); c) insulin stimulates proteoglycan synthesis in rat chondrosarcoma chondrocytes via the insulin receptor; d) mitogenic effects of insulin and MSA in human fibroblasts may be mediated by the MSA receptor, but are not mediated by the insulin receptor. However, insulin may exert mitogenic effects in other cell types via insulin receptors.

5) MSA-like polypeptides probably constitute a small portion of the total somatomedin activity of adult rat plasma, but they may represent the predominant somatomedin in fetal rat plasma. Fetal rat serum contains 20-100 times higher levels of immunoreactive MSA than does maternal or adult rat serum, suggesting that MSA may have a special role in fetal growth in the rat.

6) Insulin acting through insulin receptors also may be a critical determinant of in utero growth. Support for this view derives from demonstration of a profound reduction of insulin binding to fibroblasts from an infant with leprechaunism, intrauterine growth retardation, and marked insulin resistance (without consistent glucose intolerance). Poor third trimester growth, absent fat stores, and reduced muscle mass at birth may reflect the lack of insulin effect in fetal development that results from this heritable deficiency of insulin binding.

ACKNOWLEDGMENTS

These studies reflect the contributions and cooperation of many colleagues and collaborators, including L. Fryklund, J. Zapf, E. R. Froesch, R. E. Humbel, K. Hall, C. R. Kahn, H. Eisen, J. M. Podskalny, O. Z. Higa, C. B. Bruni, Y. Yang, V. Hascall, R. Stevens, T. Foley, J. Heaton, T. D. Gelehrter, A. M. Rosenberg, C. Grunfeld, J. J. Van Wyk, L. E.

Underwood, W. H. Daughaday, and T.-Y. Liu. We thank J. Koontz and R. Kahn for permission to discuss their results prior to publication. We thank J. Miller, J. Smith, and J. Todd for assistance in preparation of the manuscript.

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