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Structural Similarities between Human Receptors for Somatomedin C and Insulin: Analysis by Affinity Labeling†

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ABSTRACT: Human placental receptors for insulin and somatomedin C (Sm-C) were affinity labeled with [^{125}I]insulin and [^{125}I]Sm-C by using the bifunctional cross-linking agent disuccinimidyl suberate. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis demonstrated that both labeled hormones were specifically cross-linked to three protein species with apparent molecular weights of 240 000, 310 000, and 330 000. Following disulfide bond reduction, subunits of approximately 140 000 daltons were evident. Partial reduction of disulfide bonds yielded intermediate-sized species with apparent molecular weights of 180 000, suggesting the existence of an additional, smaller subunit attached to the 140 000-dalton subunit. Limited proteolysis of the hormone–receptor complexes with chymotrypsin, trypsin, and *Staphylococcus aureus* V-8 protease gave similar but not identical results for each labeled receptor. The distinction between the two receptors was further documented by inhibition of affinity labeling with graded amounts of the native hormones. These data demonstrate a substantial structural similarity between the human Sm-C and insulin receptors paralleling the homology of the native hormones and their actions.

The somatomedins (Sm's)[1] are small (approximate M_r 7500), growth hormone dependent, single-chain polypeptides which possess growth-promoting and insulin-like actions in vitro (Van Wyk & Underwood, 1978; Zapf et al., 1978; Phillips & Vassilopoulou-Sellin, 1980). At concentrations of 10^{-9}–10^{-10} M, Sm's are mitogenic for a variety of cultured cell lines, whereas 100-fold higher concentrations are required to elicit insulin-like responses. Two forms of human somatomedin have been substantially characterized. The basic form (p*I* 8.2–8.4) is known variously as somatomedin C (Sm-C) (Van Wyk et al., 1974; Svoboda et al., 1980), insulin-like growth factor I (IGF-I) (Rinderknecht & Humbel, 1978a), or "basic somatomedin" (Bala & Bhaumick, 1979). The neutral form of somatomedin has been termed insulin-like growth factor II (Rinderknecht & Humbel, 1978b).[2] Both the basic and neutral forms of Sm are structurally similar to human proinsulin (Blundell et al., 1978). Multiplication stimulating activity (MSA), a peptide isolated from the conditioned medium of rat hepatocyte cultures (Dulak & Temin, 1973; Moses et al., 1980), appears to be the rat equivalent of human IGF-II since the amino acid sequences of the two peptides are identical except for conservative substitutions at five positions (Marquardt et al., 1981).

The growth-promoting actions of Sm are thought to result from their interaction with specific somatomedin receptors, whereas the insulin-like effects are believed to occur by cross-reactivity with the insulin receptor (Clemmons et al., 1974; Van Wyk et al., 1975; Rechler et al., 1977; Zapf et al., 1981). Conversely, the mitogenic effects of insulin, usually demonstrable only at supraphysiologic concentrations, are believed to be mediated via the Sm receptor. Although cross-reactivity of Sm and insulin with their respective receptors may be due to the structural homology of the hormones, it may also reflect structural similarity of the receptors.

The structure of the insulin receptor has been studied by using a variety of methods and tissues sources (Sahyoun et al., 1978; Jacobs et al., 1979; Pilch & Czech, 1979, 1980; Wisher et al., 1980; Yip et al., 1980; Harrison & Itin, 1980; Van Obberghen et al., 1981). These data have shown that the native insulin receptor is a large (>300 000-dalton) glycoprotein composed of four disulfide-linked subunits. It appears that two identical 125 000–140 000-dalton peptide sub-

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[1] Abbreviations used: DSS, disuccinimidyl suberate; DTT, dithiothreitol; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; INS, insulin; MSA, multiplication stimulating activity; NaDodSO$_4$, sodium dodecyl sulfate; Sm, somatomedin; Sm-C, somatomedin C.

[2] Somatomedin A (Fryklund et al., 1974) is not included in this classification since this peptide remains to be fully characterized. It is described as a neutral peptide, but its biological properties are more similar to those of Sm-C/IGF-I.

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units and a pair of smaller (approximately 90000-dalton) peptide subunits are linked together to form each receptor complex.

A recent report has indicated that the human placental membrane receptor for a basic Sm is also a large glycoprotein with a 140000-dalton subunit (Bhaumick et al., 1981). The structure of the affinity-labeled MSA receptor in rat liver and human placental membranes, however, was found to differ significantly from that of the insulin receptor (Massague et al., 1981b). In the present study, we used the bifunctional cross-linking agent disuccinimidyl suberate (DSS) to covalently bind highly purified [^{125}I]Sm-C and [^{125}I]insulin to human placental membranes. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography were used to characterize and compare the specifically labeled receptors in their solubilized state, after reduction, and after partial proteolytic digestion.

Materials and Methods

Preparation of Placental Cell Membranes. Fresh human placentae from normal pregnancies were trimmed of amnion and chorion, minced and washed in cold 0.25 M sucrose, and homogenized with an Omnimixer (Sorvall) and a Polytron PT-10 (Brinkmann, setting 5 for 60 s). A membrane-rich fraction was obtained by differential centrifugation (Cuatrecasas, 1972; Marshall et al., 1974; D'Ercole et al., 1975). The protein concentration was estimated by the method of Lowry et al. (1951), and the membranes were stored at −70 °C until used.

Reagents. Human Sm-C was purified from Cohn fraction IV of human plasma as described by Svoboda et al. (1980) and iodinated with carrier-free Na^{125}I (Amersham) by using chloramine-T (Roth, 1973; Van Wyk et al., 1980). The specific activity ranged from 200 to 400 μCi/μg. Unlabeled Sm-C (specific activity at least 1000 units/mg of protein) used to assess the specificity of the receptor was derived from high-pressure liquid chromatography side fractions after isoelectric focusing (pH 7–10) and was free of insulin and IGF-II. When a large excess of unlabeled Sm-C was required for complete displacement of the radioligand, a less pure, Sm-rich extract free of immunoreactive insulin was used. Porcine insulin (Eli Lilly Co.) was iodinated with chloramine-T to a specific activity of approximately 120 μCi/μg and the iodinated peptide purified on Sephadex G-15. DSS was synthesized as previously described (Pilch & Czech, 1979; Anderson et al., 1964) and stored as a dried powder. Commercial enzyme preparations of trypsin (Worthington Biochemical Corp., 275 units/mg), chymotrypsin (Miles Biochemicals, >1000 units/mg), and *Staphylococcus aureus* V-8 protease (Miles Biochemicals, 500 units/mg) were used for proteolytic fragmentation.

Affinity Labeling. Labeling was accomplished by a modification of the procedure described by Pilch & Czech (1980). Membrane protein (500 μg) in sodium phosphate buffer (0.1 M, pH 7.4) was incubated at 4 °C with 5×10^5 cpm (~1–2 ng) of [^{125}I]Sm-C or 10^6 cpm of [^{125}I]insulin and various concentrations of unlabeled peptides in a reaction volume of 0.5 mL. After 14–16 h, DSS which had been freshly dissolved in dimethyl sulfoxide was added to give a final concentration of 20 μg/mL. After 30 min at 22 °C, the reaction was terminated by the addition of excess NH_4Br. Following the addition of 1 mL of cold phosphate buffer, the membranes were centrifuged at 9500*g* for 15 min at 4 °C. The membranes were washed with 1 mL of cold phosphate buffer and recentrifuged, and the pellet was either dissolved in gel sample solution and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis or stored at −20 °C until further analysis.

Partial Proteolytic Fragmentation. Affinity-labeled membranes were suspended in cold Tris buffer (0.05 M, pH 7.4) to give an approximate protein concentration of 10 mg/mL. Aliquots (50 μL) were then transferred to glass tubes for addition of the various proteases (concentrations in the legend of Figure 4). Proteolysis was allowed to proceed for 15 min at 22 °C and halted by the addition of $NaDodSO_4$–polyacrylamide gel electrophoresis sample solution and subsequent boiling. After the solution cooled, an equal portion of each sample was applied to a polyacrylamide gel for electrophoresis.

Polyacrylamide Gel Electrophoresis and Autoradiography. Electrophoresis was performed by using the method of Laemmli (1970). The resolving gel was 5–6.5% acrylamide and contained 0.1% sodium dodecyl sulfate. Prior to electrophoresis, the membrane pellets were boiled for 2 min in a sample solution containing 2% $NaDodSO_4$, without a reducing agent or with varied concentrations of 2-mercaptoethanol or dithiothreitol (DTT). Gels were stained with Coomassie blue and dried following destaining. Autoradiograms were made by exposing Kodak X-omat film to the dried gel in the presence of an image intensifying screen (Du Pont Cronex "Lightning plus") for approximately 7 days at −70 °C. Molecular weights were assigned by comparison to the log plot of standard marker proteins present in red blood cell membranes (Fairbanks et al., 1971).

Results

Binding Subunits and Specificity of Receptors. After reduction with 2-mercaptoethanol and $NaDodSO_4$–polyacrylamide gel electrophoresis, [^{125}I]Sm-C affinity-labeled placental membranes exhibited an intensely labeled band with an apparent molecular weight of 140000 and a faint band with an apparent molecular weight of 280000 (Figure 1, lane 1). Bands with similar mobilities were present in membranes affinity-cross-linked with [^{125}I]insulin (Figure 1, lane 3). Labeling of each receptor was abolished by incubation with high concentrations of the respective native hormones prior to cross-linking (Figure 1, lanes 2 and 4). A very small amount of specific labeling was also observed in the 90000–95000-dalton region for both receptors (Figure 4, lanes 1–4).

The specificity of the labeled Sm-C receptor was further documented by the effect on graded amounts of native Sm-C and insulin on affinity labeling (Figure 2). Labeling of both the 140000- and 280000-dalton bands was readily inhibited at concentrations of native Sm-C as low as 20 ng/mL whereas concentrations of insulin as high as 2.0 μg/mL were required to cause an equivalent decline in labeling. Conversely, with [^{125}I]insulin as the radioligand, affinity labeling was inhibited by approximately 50% with 50 ng/mL native insulin but not by 500 ng/mL native Sm-C (data not shown). The inhibitory effects of the unlabeled hormones conform to the reported relative affinities of the Sm receptor for Sm-C and insulin (D'Ercole, 1975; Van Wyk et al., 1980).

Effects of Disufide Bond Reduction on Receptors. When placental membranes affinity labeled with [^{125}I]Sm-C were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis without prior reduction, the 140000-dalton band was barely visible. Instead, three separate, although indistinct, bands were present with apparent molecular weights of 240000, 310000, and 330000 (Figure 3, lane 1). Because of the high background labeling, it is impossible to say whether a band was present at the 280000-dalton position as seen with the maximally reduced placental membranes. Alternatively, it is conceivable that the 240000-dalton nonreduced species and the 280000-dalton reduced species are indentical and only

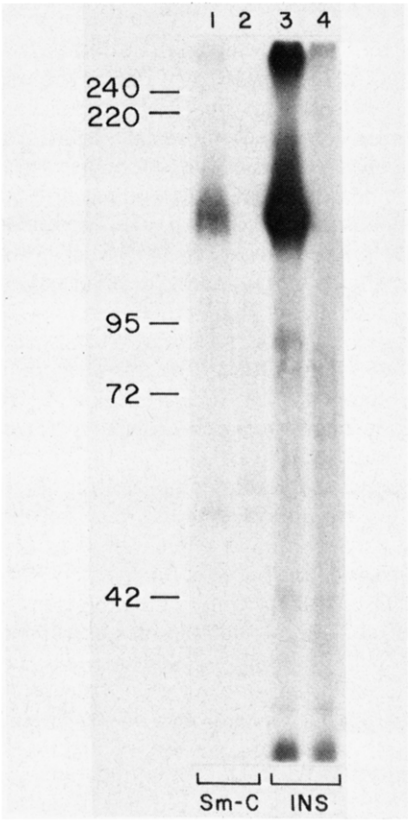

FIGURE 1: Affinity-labeled Sm-C and insulin receptors. [^{125}I]Sm-C (lanes 1 and 2) and [^{125}I]insulin (lanes 3 and 4) were affinity cross-linked to human placental membranes in the presence of (lanes 2 and 4) and absence of (lanes 1 and 3) large amounts of their respective native hormones. Samples were reduced with mercaptoethanol prior to electrophoresis on a 6.5% acrylamide gel. Numerals to the left of the lanes in this and all subsequent figures denote the positions and molecular weights ($M_r \times 10^{-3}$) of red blood cell membrane proteins. Visible bands below 72000 daltons probably represent nonspecific cross-linking since they persist in the prescence of excess native hormone. An autoradiogram of the dried gel is shown.

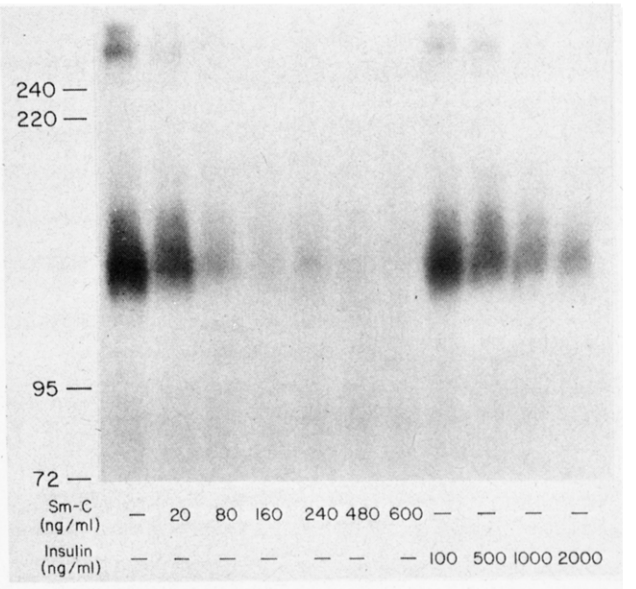

FIGURE 2: Specificity of labeled Sm-C receptor. Placental membranes were incubated with [^{125}I]Sm-C and with various concentrations of native Sm-C or insulin prior to affinity labeling. Samples were reduced with mercaptoethanol before electrophoresis on a 6.5% acrylamide gel. An autoradiogram of only the upper half of the dried gel is shown since no specific bands were present below 72000 daltons. The uppermost "band" represents material at the interface between the stacking and resolving portions of the gel.

appear different because of variable migration with NaDodSO$_4$–polyacrylamide gel electrophoresis secondary to the degree of disulfide bond reduction. Furthermore, these molecular weights must be interpreted with extreme caution since they were assigned by extrapolation to nonreduced proteins which appeared above the highest molecular weight standard.

Exposure of the labeled Sm-C receptor to low concentrations of DTT for short periods of time generated labeled bands with molecular weights of 180000 and 140000 (Figure 3, lane 5). Furthermore, increasing the concentration of reductant caused a decline in the intensity of the 180000-dalton band (Figure 3, lane 6). Similar results were obtained with the [^{125}I]-insulin-labeled membranes, although labeling was greater in the 140000-dalton region in the absence of reductant (Figure 3, lane 3) and visualization of the 180000-dalton band required a higher concentration of DTT (Figure 3, lane 7).

Results of Proteolytic Fragmentation. Susceptibility of the two labeled hormone–receptor complexes to the various proteases was similar, although the apparent sizes of the degraded fragments from each differed slightly and fewer bands were visualized upon treatment of the Sm-C receptor (Figure 4). Treatment of the labeled insulin receptor with 200 μg/mL α-chymotrypsin yielded major degradation products of approximately 125000, 110000, and 72000 daltons in addition to the nondegraded 140000-dalton subunit (Figure 4, lane 10). Identical treatment of the Sm-C receptor (Figure 4, lane 5) produced a labeled species slightly less than 140000 daltons in size and an additional degraded product with an apparent

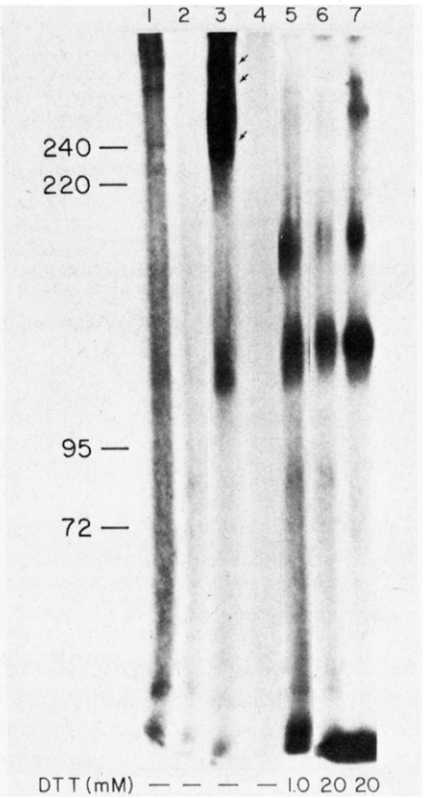

FIGURE 3: Effect of various amounts of dithiothreitol (DTT) on receptors. [^{125}I]Sm-C or [^{125}I]insulin affinity-labeled placental membranes were boiled for exactly 2 min in DTT and immediately cooled on ice until NaDodSO$_4$–polyacrylamide gel electrophoresis on a 5% acrylamide gel. The Sm-C (lane 1) and the INS receptors (lane 3) are shown in the absence of reductant and with excess native hormone (lanes 2 and 4, respectively). The arrows in lane 3 highlight bands poorly visible in the photograph. Lanes 5 and 6 show the effect of increasing the concentration of DTT on the Sm-C receptor. Lane 7 shows the partially reduced insulin receptor.

molecular weight slightly greater than 110000. Both receptors were moderately resistant to the lower concentration of trypsin

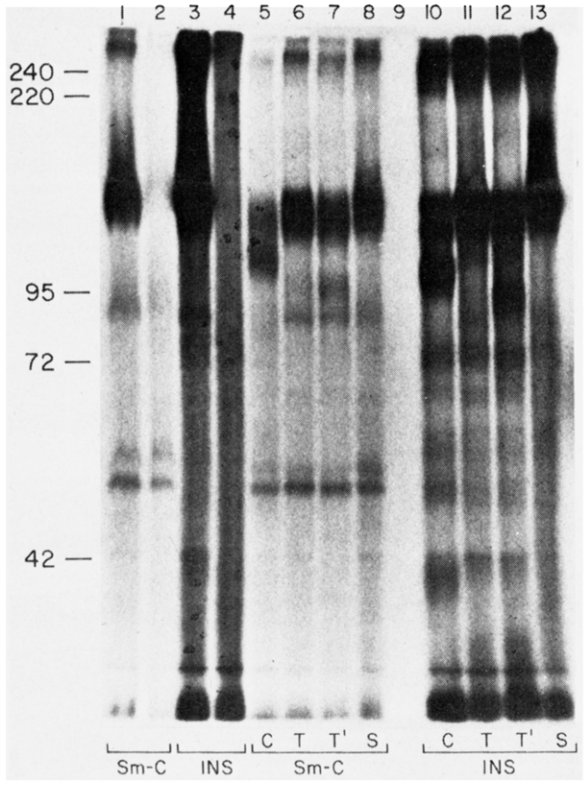

FIGURE 4: Peptidase treatment of labeled receptors. Lanes 1–4 represent untreated reduced subunits as in Figure 1. Placental membranes were affinity labeled with either [^{125}I]Sm-C (lanes 5–8) or [^{125}I]insulin (lanes 10–13) and treated with the peptidase as described under Materials and Methods. C = membranes treated with 200 μg/mL α-chymotrypsin. T and T′ = 12.5 and 25 μg/mL trypsin, respectively, and S = 22 μg/mL *Staphylococcus aureus* V-8 protease. Samples were reduced with mercaptoethanol prior to electrophoresis on a 6.5% acrylamide gel.

(12.5 μg/mL) (Figure 4, lanes 6 and 11). Following tryptic digestion of the insulin receptor with 25 μg/mL enzyme, several distinct degradation products with apparent molecular weights of 125 000, 95 000, and 72 000 were visualized as well as the 140 000-dalton nondegraded subunit (Figure 4, lane 12). Treatment of the Sm-C receptor with 25 μg/mL trypsin produced bands at 90 000, 100 000, and slightly less than 140 000 daltons (Figure 4, lane 7). Both hormone–receptor complexes were resistant to digestion by 22 μg/mL *Staphylococcus aureus* V-8 protease. Several additional bands, especially in the [^{125}I]insulin-labeled membranes, either were felt to be nonspecific because of their presence in lanes with excess native hormone or were so faint as to be of uncertain significance.

Discussion

Previous studies indicate that the insulin receptors from a number of tissues, including human placenta, are disulfide-linked heterotetramers composed of two α (approximate M_r 125 000–140 000) and two β (approximate M_r 90 000) subunits (Jacobs et al., 1979, 1980a; Massague et al., 1980, 1981a). Mild treatment with a disulfide reducing reagent generates disulfide-linked dimers, and further reduction yields isolated subunits. The β subunit is readily cleaved by endogenous proteases to a 45 000-dalton peptide (β_1), and the resulting partially cleaved complexes probably account for the heterogeneity of size of the nonreduced receptor in placental membrane preparations (Massague et al., 1981a). Affinity labeling techniques predominately label the α subunit (Jacobs et al., 1979; Yip et al., 1980; Wisher et al., 1980; Pilch & Czech, 1979).

The results of the present study concur with this proposed structure of the insulin receptor. In addition, they indicate that the Sm-C receptor has a very similar subunit structure. The affinity-labeled human Sm-C receptor also appears to be a large complex composed of disulfide-linked peptides. Although the appearance of at least three distinct cross-linked species in the unreduced state may represent binding to separate receptors or cross-linking to other membrane proteins, it more likely reflects limited proteolysis of intact receptors as previously shown for the insulin receptor (Massague et al. 1981a). The small amount of 140 000-dalton material present after electrophoresis of the nonreduced receptors may be the reduced 140 000-dalton subunit or a proteolytic fragment of the intact receptor complex. The predominant labeling of the 140 000-dalton subunit suggests that this region contains the portion of the receptor responsible for ligand binding. This subunit corresponds to the α subunit of the insulin receptor and in the present study exhibited identical migration on $NaDodSO_4$–polyacrylamide gel electrophoresis. The small amount of specific labeling at approximately 90 000 daltons after reduction could represent either labeling of a β subunit or degradation of the 140 000-dalton species. However, the generation of a 180 000-dalton labeled species, analogous to the $\alpha\beta_1$ dimer reported for the insulin receptor, under conditions of partial reduction of disulfide bonds implies that there is a disulfide-linked subunit with an approximate molecular weight of 40 000 daltons not directly bound to the labeled Sm-C.

Following maximal reduction of the disulfide bonds of both receptors, we consistently observed a small amount of radioactivity in the 280 000-dalton region of the gel. This was also noted by Pilch & Czech (1980) when they used DSS to affinity label the insulin receptor. Since DSS nonspecifically cross-links peptides through free amino groups, this high molecular weight complex may represent covalent linkage of the 140 000-dalton complex with another membrane protein or a dimer of two 140 000-dalton subunits. Alternatively, this may reflect cross-linking of the radioligands to the 255 000-dalton MSA receptor (Massague et al., 1981b).

Limited proteolysis of the cross-linked insulin receptor yielded labeled fragments remarkably similar to those obtained by Jacobs et al. (1980a). Similar treatment of the Sm receptor produced comparable fragments and provides further evidence for the structural similarity of the two subunits. The decreased number of identifiable fragments upon proteolysis of [^{125}I]-Sm-C-labeled membranes may be due to the less intense initial labeling obtained relative to membranes labeled with [^{125}I]-insulin.

Although little substantial structural difference between the two receptors was found in this study, the receptors are clearly delineated by their differing affinities and the slight variation in size of their respective proteolytic fragments. Moreover, a previous report has shown that antibodies to a purified insulin receptor react poorly, if at all, with an affinity-labeled Sm receptor (Bhaumick et al., 1981). More refined techniques than those currently available will be required to determine what structural variance is responsible for their differing specificities and affinities.

Our findings concur with the studies of Bhaumick et al. (1981), who photoaffinity labeled human placental membranes with a derivative of radiolabeled basic Sm (4-azido-2-nitrophenyl[^{125}I]Sm). These authors reported the labeled receptor to be a large, apparently glycosylated protein which yielded a 140 000-dalton labeled subunit upon reduction of disulfide bonds. In contrast, Massague et al. (1981b) used DSS to label

rat liver and adipocyte and human placental receptors with ^{125}I-labeled MSA and found a binding species with an apparent molecular weight of 255 000 that did not consist of disulfide-linked subunits. This observation is surprising since MSA and its human counterpart, IGF-II, are more insulin-like in adipose tissue assays and more cross-reactive with the insulin receptor than is Sm-C/IGF-I (Van Wyk et al., 1975; Zapf et al., 1978, 1981).

Although the structural homology of the Sm-C/IGF-I and insulin receptors is clear, too few peptide hormone receptors have been comparably studied to define the singularity of this observation. The receptor size and subunit structure for certain other peptide hormones, however, have been reported to differ substantially from those of insulin and Sm-C. The rabbit growth hormone receptor is a large glycoprotein, but upon reduction, its major band is approximately 75 000–85 000 daltons (Waters & Friesen, 1979). The human placental membrane receptor for epidermal growth factor has a molecular weight of approximately 180 000 and is unaffected by disulfide bond reduction (Das et al., 1977; Hock et al., 1980; O'Keefe et al., 1981). Finally, the rat hepatic glucagon receptor has recently been described as a single-chain, 53 000-dalton peptide (Johnson et al., 1981).

The presence of Sm-C-like substances in submammalian species (Furlanetto et al., 1977; Rothstein et al., 1980) and the decreased homology between human IGF-I and the insulin of lower vertebrates have suggested that these hormones may have evolved from a common ancestral peptide (Rinderknecht & Humbel, 1978a). The present studies now show that the respective receptors for insulin and Sm-C/IGF-I also display structural homology. Further studies in lower species, therefore, might delineate whether differentiation of receptor specificity paralleled the mutations responsible for the changes in peptide structure and function. These and related techniques should be useful for further investigation of the ultimate fate of the hormone–receptor complex within the cell and the role of this complex in hormone action.

Added in Proof

After submission of the manuscript, Kasuga et al. (1981) reported the affinity labeling of BRL 3A2 rat liver cells with [^{125}I]IgF-I. The resultant hormone–receptor complex had an apparent molecular weight >300 000 (nonreduced). After disulfide bond reduction, an apparent receptor subunit with a molecular weight of approximately 130 000 was observed.

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Aggregation Competence of Proteoglycans from the Substratum Adhesion Sites of Murine Fibroblasts†

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ABSTRACT: Supramolecular, proteoglycan-containing complexes from the serum-coated tissue culture substratum adhesion sites of Balb/c and Swiss 3T3 murine fibroblasts have been characterized after [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid mediated detachment of cells, extraction of the substratum-bound sites with 4 M guanidine hydrochloride (Gdn·HCl), and reassociation of proteoglycan-containing complexes by dialysis against 0.4 M Gdn·HCl. Sepharose CL2B gel filtration of extracts under associative conditions demonstrates a large proportion of aggregated proteoglycan material, some of which resists dissociation with 4 M Gdn·HCl and requires a detergent for complete dissociation. Isopycnic CsCl density gradient centrifugation of long-term-radiolabeled adhesive material under associative conditions reveals radiolabeled bands at three densities. A small fraction of two bands can be shown to be aggregates by exclusion from Sepharose CL2B columns and their dissociation with detergent. Digestion of the gradient bands with selective glycosaminoglycan-degrading enzymes documents that (a) the highest buoyant density band contains primarily heparan sulfate and chondroitin sulfate, with possibly a small amount of hyaluronic acid; (b) the mid-density band is comprised only of hyaluronate; and (c) the lightest band is a complex mixture of hyaluronate, chondroitin sulfate, and glycoprotein. The proteoglycan nature of some of the material in the gradient bands is shown by its sensitivity to Pronase and its lability to alkaline sodium borohydride reduction. Isopycnic density gradient analyses of Gdn·HCl extracts of newly formed footpad adhesion sites under associative conditions are qualitatively similar to those described above for long-term-generated adhesive material (enriched in "footprint" material as a result of natural cell movement across the substratum). However, there are significant quantitative and density gradient behavioral differences. These studies indicate that (a) some of the proteoglycan material from fibroblast cell–substratum adhesion sites is competent to form supramolecular aggregates, (b) there appear to be multiple types of aggregates which differ in several properties from cartilage-like hyaluronate/chondroitin proteoglycan aggregates, (c) the adhesion sites of both cell types have proteoglycans with similar properties, and (d) the proteoglycan material in newly formed adhesion sites is different from that in long-term-generated adhesion sites.

The survival of most mammalian cells in culture depends upon their ability to attach and adhere to a suitable tissue culture substratum, much the way cells in vivo attach to an extracellular matrix. Fibroblasts attach to a serum-coated substratum with both very tightly apposed focal regions and broad "close" contact regions (Izzard & Lochner, 1976, 1980; Heath & Dunn, 1978; Couchman & Rees, 1979). The tightly apposed focal regions have been called "footpads" upon visualization with the scanning electron microscope (Revel et al., 1974; Rosen & Culp, 1977; Vogel, 1978; Britch & Allen, 1980). Fibroblasts that are motile in culture appear to break off from footpads at their posterior or trailing edge and make new ones directly behind the ruffling anterior membrane. The footpad adhesion sites thus left behind are referred to as "footprint" material (Culp, 1976, 1978; Chen, 1977).

Exposure of fibroblasts to the calcium-specific chelator [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA)[1]

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[1] Abbreviations used: C0S, unsulfated chondroitin; C6S, chondroitin 6-sulfate; C4S, chondroitin 4-sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GAG, glycosaminoglycan; Gdn·HCl, guanidine hydrochloride; HA, hyaluronic acid; HS, heparan sulfate; L-SAM, long-term metabolically radiolabeled substratum-attached material containing both footpad and footprint adhesive material; MEM × 4, Eagle's minimal essential medium supplemented with a 4-fold concentration of vitamins and essential amino acids; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline without divalent cations; PMSF, phenylmethanesulfonyl fluoride; R-SAM, reattaching substratum-attached material containing newly formed footpad adhesion sites; ΔDi-6S, ΔDi-4S, and ΔDi-0S, $\Delta^{4,5}$-unsaturated disaccharides liberated by chondroitinase digestion of chondroitin 6-sulfate, chondroitin 4-sulfate, and unsulfated chondroitin, respectively; DS, dermatan sulfate.

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