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MONOCLONAL ANTIBODIES AS PROBES OF INSULIN RECEPTOR STRUCTURE AND FUNCTION

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ENDOCRINOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

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"S'il fallait donner une comparaison qui exprimât mon sentiment sur la science de la vie, je dirais que c'est un salon superbe tout resplendissant de lumière dans lequel on ne peut parvenir qu'en passant par une longue et affreuse cuisine."

Claude Bernard

Introduction à l'Étude de la

Médicine Expérimentale

ACKNOWLEDGEMENTS

Countless friends and family members have provided inspiration or contributed in some way to the work presented in this dissertation. A few of these people deserve my special thanks. In particular, it is with deep respect and admiration that I acknowledge the brilliant guidance of Richard Roth throughout the course of these studies. I am also deeply indebted to my parents, Owen and Sadie Morgan, whose generous support has never faltered during the past 27 years. I also wish to thank Richard Weiner, Ira Goldfine, and Vishwanath Lingappa for their guidance. Finally, I thank Andrea Weisman, Jim Flannery, Mike Talman, Tom Ahrens, John Forsayeth, Dennis McChesney, and my other friends in San Francisco for their encouragement.

Certain chapters of this dissertation are reprints of material as it appears or will appear in *Endocrinology* (Chapter 1), *The Proceedings of the National Academy of Sciences USA* (Chapter 2), and *Biochemistry* (Chapters 3,4). Where necessary, permission to reprint the material was obtained from the copyright owner. The last coauthor listed in these publications directed and supervised the research which forms the basis for the dissertation. I wish to gratefully acknowledge the assistance of Lisa Ho and Lawrence Korn, who are listed as coauthors in Chapter 2 for performing oocyte injections and oocyte maturation analyses, and Kurt Jarnagin, who is listed as a coauthor in Chapter 4 for initiating the IGF-I receptor experiments by providing the large numbers of BRL-3A cells needed.

Abstract:

MONOCLONAL ANTIBODIES AS PROBES OF INSULIN RECEPTOR STRUCTURE AND FUNCTION

David Owen Morgan

Insulin initiates its diverse anabolic responses by binding to its membrane receptor, a disulfide linked tetramer of two α subunits and two β subunits. The binding of insulin to the α subunit activates a tyrosine-specific protein kinase in the cytoplasmic domain of the β subunit. The role of this receptor kinase in insulin action is not clear; indeed, there is some evidence that the kinase does not mediate certain rapid insulin actions. Thus, the present studies were performed in order to characterize the structure and function of the insulin receptor kinase and clarify its role in the mechanism of insulin action. The experimental strategy chosen for this purpose involved the development of a panel of monoclonal antibodies to the insulin receptor kinase. The production of these reagents began with the development of a rapid and sensitive method for identifying hybridoma cells producing monoclonal anti-receptor antibodies. This method was then used successfully in the production of a panel of 36 monoclonal antibodies to the human insulin receptor. 34 of these antibodies recognized the receptor's cytoplasmic kinase domain, and studies with receptors from various species indicated that the antigenic structure of these regions has been highly conserved during evolution. Antigenic mapping studies demonstrated that the receptor kinase domain contains at least 4 major antigenic regions, which were here named the β_1 to β_4 regions. The β_2 region was found to be particularly important in kinase function since antibodies to this region almost completely inhibited receptor autophosphorylation and kinase activity. In addition, the β_2 antibodies were found to fully recognize and inhibit the kinase activity of the closely related receptor for insulin-like

growth factor-I (IGF-I). The close relationship between the insulin and IGF-I receptors was then further demonstrated in studies of purified IGF-I receptor prepared by immunoaffinity chromatography with these antibodies. Finally, the β_2 antibodies were used to approach the question of the importance of the kinase in insulin action. Two techniques (microinjection and osmotic lysis of pinosomes) were used to introduce an inhibitory antibody into the cytoplasm of various cell types. The resulting inhibition of receptor kinase activity *in vivo* was found to inhibit insulin's stimulation of: (i) frog oocyte maturation; (ii) glucose uptake by CHO cells, TA1 adipocytes, and primary rat adipocytes; (iii) ribosomal protein S6 phosphorylation in CHO cells; and (iv) glycogen synthesis in HepG2 cells. In TA1 cells, the stimulation of glucose uptake by the IGF's was also inhibited. Thus, receptor kinase activity was found to play a role in various rapid, intermediate, and long-term insulin actions in a broad range of cell types.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

(continued next page)

LIST OF FIGURES (continued)

INTRODUCTION

Metabolism involves a balance of two opposing processes: catabolism, the oxidative breakdown of carbohydrates, fats, and proteins to release energy; and anabolism, the biosynthesis of these large energy-rich substances from smaller compounds. In multicellular organisms, the broad range of metabolic processes occurring in various body systems must be carefully integrated to meet the demands of a constantly changing environment. This integration of metabolism requires efficient methods of intercellular communication. Thus, the endocrine system, with its ability to coordinate distant body systems through the use of blood-borne messengers, or hormones, plays an important part in metabolic integration. Insulin, glucagon, epinephrine, and norepinephrine are particularly important hormones in this regard (Ganong, 1985; Stryer, 1981), and it is largely their coordinated actions which allow the organism to maintain the required balance between opposing catabolic and anabolic processes.

Insulin is the major anabolic and anti-catabolic hormone, and elicits an extraordinary array of responses whose general outcome is the storage of excess fuels in the form of various complex macromolecules. Since the actions of insulin are remarkably diverse as well as important, they are of interest as an intriguing model system for the study of hormone action. Furthermore, insulin is of major importance in human medicine (Kahn, 1983, 1985). A variety of common metabolic disorders occur as a result of abnormalities in insulin secretion and insulin action, and many of these disorders, including the various forms of diabetes mellitus, are fatal if untreated. Thus, insulin and insulin action have been the subjects of intense investigation since the hormone was discovered over 60 years ago.

Recently, this research has centered on the molecular mechanisms underlying insulin action. Such studies have clarified the structure of insulin's cell surface receptor protein and have revealed the effects of insulin on several metabolic processes and enzyme activities. However, the missing link in these studies has been the mechanism by which the insulin

receptor activates these changes inside the cell. This process of signal transmission by the insulin receptor is a major theme in the present work. The following pages provide a brief review of insulin action and outline some of the proposed mechanisms of insulin action. In subsequent chapters, studies will be presented in which monoclonal anti-receptor antibodies were developed and used as tools to clarify the molecular events involved in transmembrane signal transmission by the insulin receptor.

Insulin action

Insulin's various anabolic and anti-catabolic effects can be conveniently grouped according to how rapidly they occur: generally, they can be divided into rapid (seconds to minutes), intermediate (minutes to hours), and long-term (hours to days) effects (Ganong, 1985; Goldfine, 1981; Kahn et al., 1981). Although the following discussion is restricted to the three major insulin target organs (muscle, fat, and liver), the effects discussed here are also observed in a broad range of other cell types.

One of insulin's most well known and most rapid actions is its ability to stimulate glucose transport across the cell membrane (Cushman et al., 1984; Kono, 1984). This stimulation of glucose transport occurs predominantly in muscle and fat cells, occurs within seconds of insulin treatment, and is mediated by the glucose transporter, an integral membrane protein (M_r=40 to 60 K) whose primary structure has recently been determined (Mueckler et al., 1985). Several studies by Cushman, Kono, and colleagues have demonstrated that insulin elicits its effects on glucose transport by stimulating the translocation of glucose transporters from an intracellular pool to the plasma membrane (Cushman et al., 1984; Kono, 1984). Insulin also stimulates a variety of other membrane transport systems, including those responsible for the uptake of amino acids by muscle cells. In both muscle cells and adipocytes, the transport of potassium and other ions is also altered, resulting in changes in membrane potential .

The stimulation of membrane transport provides the building blocks required to supply the increased biosynthetic processes of the insulin-stimulated muscle or fat cell. The major intermediate effect of insulin on these cells is the stimulation of various metabolic enzymes inside the cell (Denton et al., 1981; Goldfine, 1981). In adipocytes, these effects result in increased fatty acid synthesis, increased glycerol phosphate synthesis, increased triglyceride deposition, increased lipoprotein lipase activity, and decreased lipolysis by hormone-sensitive lipase. In muscle, the synthesis of both glycogen and protein are stimulated, while the opposing processes (glycogenolysis, gluconeogenesis, and protein degradation) are inhibited.

Liver cell metabolism is also profoundly altered by insulin. Although membrane transport of glucose occurs freely and is not regulated by insulin in the liver cell, this cell type does exhibit intermediate insulin effects on enzyme activities similar to those seen in muscle and fat. Thus, the synthesis of glycogen, lipids, and protein are all stimulated in the liver cell by insulin, and these effects, combined with decreased gluconeogenesis and increased glycolysis, lead to a major decrease in glucose output by the liver.

Long term actions of insulin include the stimulation of RNA and DNA synthesis and the promotion of cellular growth and differentiation. Many of these effects often require high concentrations of insulin, and may result from the interaction of insulin with the closely related receptor for insulin-like growth factor-I (Straus, 1984; Froesch et al., 1985). The close relationship between the actions of insulin and insulin-like growth factor-I will be discussed in later sections.

The insulin receptor

Insulin's broad range of biological effects are initiated by the interaction of the hormone with a specific receptor protein on the surface of target cells. The insulin-binding properties of this receptor were established early in the development of radiolabeled insulin binding techniques (for review, see Gammeltoft, 1984), and more recently the focus of insulin

receptor research has centered on its molecular structure and function (Czech, 1985; Kahn, 1985). A wide range of techniques have demonstrated that the receptor is composed of two α (M_r=135,000) and two β (M_r=95,000) subunits in a disulfide linked heterotetramer $(\alpha\beta)_2$ (Jacobs and Cuatrecasas, 1981). Both subunits are glycosylated (Hedo et al., 1981) and exposed to the extracellular environment, but only the β subunit is exposed to the cytoplasm (Hedo and Simpson, 1984; Ebina et al., 1985b; Ullrich et al., 1985). The α subunit can be labeled with photoaffinity insulin analogues (Yip and Moule, 1983; Wisher et al., 1980) and affinity cross-linking techniques (Pilch and Czech, 1979), and therefore appears to be the major site of insulin binding. In intact cells, the binding of insulin causes the rapid phosphorylation of the β subunit on serine and tyrosine residues (Kasuga et al., 1982b,d; Haring et al., 1984). Serine phosphorylation of the receptor is not seen in purified receptor preparations (Kasuga et al., 1983a; Roth and Cassell, 1983), and is therefore mediated by an insulin-sensitive protein kinase which is not tightly associated with the receptor. On the other hand, the following lines of evidence indicate that receptor phosphorylation on tyrosines is mediated by a tyrosine-specific protein kinase located in the receptor β subunit. First, receptors purified to near homogeneity still possess insulin-stimulated tyrosine kinase activity and autophosphorylation sites (Kasuga et al., 1983a; Petruzzelli et al., 1982; Roth and Cassell, 1983). In addition, the β subunit is labeled by ATP affinity derivatives (Roth and Cassell, 1983; Shia and Pilch, 1983; Van Obberghen et al., 1983), and specific proteolysis of the β subunit abolishes the kinase activity of the receptor without affecting insulin binding activity (Roth et al., 1983). Finally, recent studies of the amino acid sequence of the receptor, deduced from cloned cDNA sequences, indicate that the cytoplasmic domain of the β subunit contains sequences homologous to the ATP binding site and autophosphorylation sites of other tyrosine kinases (Ebina et al., 1985b; Ullrich et al., 1985). These recent studies have demonstrated that the insulin receptor is a member of a large family of tyrosine-specific protein kinases made up by various viral transforming proteins and the receptors for several growth factors (epidermal

growth factor, EGF; platelet derived growth factor, PDGF; insulin-like growth factor-I, IGF-I)(Heldin and Westermark, 1984; Hunter and Cooper, 1985).

The insulin-stimulated autophosphorylation of the β subunit on tyrosine residues is apparently an intramolecular reaction (Petruzzelli et al., 1984; Shia et al., 1983; White et al., 1984) which results in an increase in the kinase activity toward exogenous substrates (Rosen et al., 1983; Yu and Czech, 1984). In partially purified receptor preparations, the enhanced activity of this autophosphorylated kinase is retained even after the dissociation of insulin by mildly acidic treatment (Rosen et al., 1983), and only subsequent dephosphorylation with alkaline phosphatase results in a decrease in kinase activity (Yu and Czech, 1984). In the intact cell, dephosphorylation is rapidly accomplished by cellular phosphatases (Haring et al., 1984). These results suggest that the role of autophosphorylation is to provide a reversible activation of the tyrosine kinase activity.

The insulin receptor, like all cellular proteins, passes through a dynamic life cycle involving biosynthesis and degradation. Studies of metabolically labeled cells demonstrate that the receptor is synthesized as a glycosylated precursor with a molecular weight of 190 to 200 kDa under reducing conditions (Deutsch et al., 1983; Hedo et al., 1983; Kasuga et al., 1982a). Further glycosylation and cleavage of this precursor results in the mature α and β subunits found on the cell surface (Forsayeth et al., 1986; Hedo et al., 1983; Ronnett et al., 1984). Recent studies of the receptor's amino acid sequence also indicate that the receptor originates as a single-chain pro-receptor (1382 amino acids) which encodes both the α subunit (735 amino acids, 84.2 kDa) and the β subunit (620 amino acids, 69.7 kDa)(Ebina et al., 1985b).

Degradation of the insulin receptor occurs with a half-life of about 7 to 12 hours (Kasuga et al., 1981; Reed et al., 1981), and is apparently mediated by receptor internalization. Furthermore, several studies have demonstrated that insulin greatly increases the rate of receptor internalization and degradation (Kasuga et al., 1981; Ronnett et al., 1982). This process, known as receptor down-regulation, allows insulin to decrease

target cell sensitivity by reducing the cell surface levels of its receptor (Gavin et al., 1974). Since other investigations have indicated that most internalized receptors actually escape degradation and are recycled to the cell surface (Fehlmann et al., 1982; Marshall and Olefsky, 1982), receptor internalization is probably important in other aspects of insulin action as well. For example, internalization may be required to bring the activated receptor kinase inside the cell to a location where it can interact with the appropriate substrates.Similarly, the internalization of insulin itself may allow the hormone to interact with putative receptors on intracellular organelles such as the nucleus (Goldfine et al., 1982). In addition, receptor-mediated endocytosis of insulin may be important in the degradation of the hormone (Terris and Steiner, 1975).

The molecular mechanism of insulin action

The binding of insulin to its receptor causes alterations in a remarkable array of cellular functions. Clearly, the initial receptor activation event must be amplified through a variety of divergent molecular pathways to be capable of influencing such a broad range of enzyme activities. The nature of these pathways is gradually emerging.

To begin with, it has long been clear that insulin treatment rapidly alters the serine phosphorylation state of a variety of proteins, including several crucial metabolic enzymes. For example, insulin treatment causes the dephosphorylation of glycogen synthetase and pyruvate dehydrogenase, and these dephosphorylation events lead to the appropriate increase in the activity of both enzymes (Denton et al., 1981). On the other hand, insulin causes increased phosphorylation of ATP citrate lyase, ribosomal protein S6, and acetyl-CoA carboxylase, although the activity of only the latter enzyme is clearly increased by these phosphorylations (Avruch et al., 1982). Indirect evidence also supports a role for dephosphorylation in the effect of insulin on the activities of several other enzymes involved in glycogen metabolism (phosphorylase kinase, phosphorylase), glycolysis (pyruvate kinase), and lipid metabolism (hydroxymethyl glutaryl CoA reductase,

triacylglycerol lipase)(Denton et al., 1981). Similarly, insulin's effect on cyclic AMP metabolism (increased phosphodiesterase activity) may be mediated by increased phosphorylation (Marchmont and Houslay, 1980).

As mentioned earlier, the insulin receptor itself is also a major substrate for serine-specific protein kinases (Kasuga et al., 1982b,d; Gazzano et al., 1983), whose identity remains largely a mystery (Haring et al., 1985; see below). Insulin-stimulated serine phosphorylation of the receptor may provide the framework for some as yet unidentified feedback mechanism. Interestingly, recent studies suggest that non-insulin-dependent receptor phosphorylation by cyclic AMP-dependent kinase (Stadtmauer and Rosen, 1986) or protein kinase C (Bollag et al., 1986; Jacobs et al., 1983c; Takayama et al., 1984) may lead to modifications in the receptor tyrosine kinase activity. However, there is no direct evidence to suggest that insulin-stimulated receptor phosphorylation on serines is accompanied by changes in receptor kinase activity (Czech, 1985).

In any case, it is clear from these various studies of protein phosphorylation that insulin action involves a potentially complex pattern of activation and/or inhibition of serine-specific protein kinases and/or phosphatases. One clue to the nature of this complex system comes from evidence that insulin causes the dephosphorylation and inhibition of phosphatase inhibitor-1, which normally inhibits the activity of the general protein phosphatase (Nemenoff et al., 1983). In addition, several workers have identified insulin-sensitive serine kinases which phosphorylate ribosomal protein S6 (Donahue and Massaracchia, 1984; Perisic and Traugh, 1983; Tabarini et al., 1985). One of these S6 kinases may be a substrate for the insulin receptor (Roth et al., 1985), while another may be capable of phosphorylating the receptor (Tuazon et al., 1985). In other searches for receptor-activated serine kinases, Haring et al. (1985) found that the partially purified insulin receptor did not phosphorylate a wide range of kinases known to phosphorylate glycogen synthetase, although they did find some evidence that the receptor might phosphorylate the Ca^{++}/calmodulin-dependent kinase.

Thus, the manner in which the insulin receptor activates protein phosphorylation pathways is not well understood. Several mechanisms may be involved, and one of these may involve the insulin-dependent generation of some chemical second messenger or 'mediator' by the plasma membrane. Such mechanisms are appealing since they allow for great amplification of the initial insulin signal, but the search for these mediators of insulin action has been a long and generally fruitless one. To begin with, it seems clear that the classical second messengers involved in the actions of other hormones (cyclic AMP, calcium, membrane potential) are not fundamentally important in insulin action (Denton et al., 1981; Kahn, 1985). Several groups have reported the release from the membrane of other, perhaps peptidic, substances capable of regulating certain insulin-sensitive enzymes; however, despite intense effort, the chemical identity of these substances has not been elucidated (Seals and Jarett, 1980; Larner et al., 1979; Seals and Czech, 1981; Jarett et al., 1982; Cheng and Larner, 1985). Recently, Saltiel et al. (1986) have reported that insulin may stimulate an endogenous phospholipase C activity that hydrolyzes a novel glycolipid to generate a complex carbohydrate-phosphate substance capable of mediating some insulin actions. The importance of this mediator remains to be seen.

The discovery of the receptor-associated tyrosine kinase activity has provided a major breakthrough in the search for the elusive mechanism of insulin action. The activation of the receptor kinase fulfills the requirements for a universal signal transmission event capable of initiating a broad range of pathways, many of which involve protein phosphorylation (see above). Furthermore, kinase activation is clearly a very early step in the insulin action pathway, both in terms of its rapid onset (Pang et al., 1985; White et al., 1984) and its physical location within the receptor molecule. In fact, it is the only enzymatic activity known to be located within the receptor molecule and apparently takes up the major portion of the receptor's cytoplasmic domain (Ebina et al., 1985b, Ullrich et al., 1985). Thus, it is tempting to speculate that kinase activation is responsible for initiating insulin action, probably through a variety of secondary molecular events which may include the

generation of the aforementioned 'mediators' and the activation of serine kinases or phosphatases.

How might tyrosine kinase activation lead to signal transduction? The most likely possibility is simply that the kinase phosphorylates and activates endogenous substrates responsible for eliciting subsequent steps in insulin action. Several potential substrates have thus far been reported in insulin treated cells, although at this point their only known characteristic is their mobility on polyacrylamide gels (M_r's of 120, 185, 46, and 160 K) (Rees-Jones and Taylor, 1985; White et al., 1985; Haring et al., 1986; Yu et al., 1986). On the other hand, the receptor kinase may not act through a substrate; instead, the autophosphorylation of the receptor itself may be the signalling event, such that autophosphorylation leads to a change in some other receptor activity or the noncovalent interaction of the receptor with some other effector molecule.

Although the receptor kinase could theoretically mediate all of insulin's actions, there is still some question regarding its actual role. Several lines of indirect evidence support a role for the kinase in insulin action (see Chapter 5), but studies have also been reported which suggest that the kinase is not important, particularly in rapid insulin effects. Briefly, these studies showed that certain human patients' sera, which contain antibodies to the receptor, are capable of stimulating glucose uptake by adipocytes without stimulating receptor phosphorylation (Simpson and Hedo, 1984; Zick et al., 1984a). Thus, a definite role for the kinase has not yet been established (The question of the role of the kinase will appear regularly in subsequent sections of this dissertation, and arguments for and against the importance of the kinase will be presented to some extent in Chapter 2 and in great detail in Chapter 5).

Relationships between insulin and insulin-like growth factors

A clear understanding of insulin action requires some knowledge of a closely related pair of peptides known as insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II

(IGF-II), which were originally identified on the basis of their ability to mimic insulin action in certain situations (Zapf et al., 1981). Both of these factors possess amino acid sequences and three-dimensional structures which are homologous to those of insulin (Blundell and Humbel, 1980). Similarly, the three hormones interact to some extent with each other's receptors (Rechler and Nissley, 1985; Froesch et al., 1985). Thus, insulin binds with high affinity to its own receptor, with moderate affinity to the IGF-I receptor, and does not interact significantly with the IGF-II receptor. IGF-I, on the other hand, binds with high affinity to its own receptor and with moderate affinity to both the insulin and IGF-II receptors. Finally, IGF-II binds with high affinity to its own receptor, with moderate affinity to the IGF-I receptor , and with low affinity to the insulin receptor. Not surprisingly, it is often difficult to determine exactly which receptor is responsible for the actions of each of these hormones. For example, as mentioned earlier, many of insulin's long term effects on cell growth and differentiation require high insulin concentrations, and therefore may be mediated by the IGF-I receptor (Straus, 1984). Similarly, many of the rapid actions of IGF-I on metabolic processes may be mediated by the insulin receptor (Froesch et al., 1985).

The close relationship between insulin and IGF-I is further exemplified by a consideration of the structure and function of their receptors. Although the IGF-II receptor is apparently unrelated to the insulin receptor (it is a monomer [M_r=260,000] without tyrosine kinase activity)(Corvera et al., 1986), the receptors for insulin and IGF-I are remarkably similar. The IGF-I receptor exhibits an $(\alpha\beta)_2$ tetrameric structure involving an α subunit which binds the ligand and a β subunit which apparently contains a tyrosine-specific protein kinase activity (Rechler and Nissley, 1985; see Chapter 4). The inability to extensively purify the IGF-I receptor has made it difficult to rigorously characterize certain aspects of its structure and kinase activity, as will be seen in studies presented in Chapter 4.

Antibodies as probes of insulin receptor structure and function

A great number of the concepts discussed in this introduction were formulated on the basis of experiments performed with antibodies to the insulin receptor. These antibodies have been immensely valuable tools throughout the recent course of insulin receptor research. In fact, anti-receptor antibodies form the underlying framework upon which all of the studies presented in this dissertation are based.Therefore, it is instructive to briefly review the role that antibodies have played in the study of insulin action.

Polyclonal antibodies to the receptor, isolated from patients with the type B syndrome of extreme insulin resistance and acanthosis nigricans, have been instrumental in probing the structure of the receptor and its relationship to hormone action (Kahn et al., 1981; De Pirro et al., 1984). These antibodies can immunoprecipitate the receptor from a variety of metabolically labeled cells, providing information about receptor structure (Van Obberghen et al., 1981), biosynthesis (Hedo et al., 1983), degradation (Kasuga et al., 1981), and phosphorylation (Kasuga et al., 1982b,d). Since they mimic several insulin effects and inhibit the binding of insulin to the receptor, they can be used to clarify the relationship between insulin binding and insulin action (Baldwin et al., 1980; Kahn et al., 1981). As mentioned earlier, the ability of some of these antibodies to stimulate insulin action without stimulating receptor phosphorylation has challenged the notion that the kinase mediates insulin action (Simpson and Hedo, 1984; Zick et al., 1984a). Some of these antibodies are also capable of mimicking insulin's ability to down-regulate the receptor, and thus have been useful in revealing clues to the nature of this process (Grunfeld, 1984; Taylor and Marcus-Samuels, 1984).

Nevertheless, there are several limitations to the use of these antibodies. They are available in extremely limited amounts, and their titers and affinities are often relatively low. Most importantly, these antisera are polyclonal and therefore contain mutiple populations of antibodies (De Pirro et al., 1984), some of which may recognize proteins

other than the receptor. To circumvent these limitations, considerable effort has recently been devoted to the development of monoclonal antibodies to the insulin receptor.

A monoclonal antibody is secreted by a cloned hybrid cell line formed originally by the fusion of a lymphocyte and a myeloma cell (Kohler and Milstein, 1975). This hybridoma cell has the immortality of the myeloma cell and secretes the single specific antibody of the lymphocyte. Thus, monoclonal antibodies have the distinct advantage of being available in essentially unlimited amounts and being specific for a single antigenic determinant.

Monoclonal antibodies have been useful in studies of the insulin receptor (Roth et al., 1982; Kull et al., 1983; Ganguly et al., 1985; Soos et al., 1986). Like the polyclonal antisera mentioned above, these monoclonal antibodies can be used to immunoprecipitate the receptor in studies of receptor structure (Roth et al., 1982; Kull et al., 1983; Ellis et al., 1986), turnover (Jacobs et al., 1983a), and phosphorylation (Roth et al., 1983a; Ellis et al., 1986). They are also useful for developing immunoaffinity columns for use in purifying the insulin receptor from various sources (Roth and Cassell, 1983; Roth et al., 1985; Morgan et al., 1986a,b). Due to their high specificity, monoclonal antibodies can be used to map antigenic regions (Soos et al., 1986) and can detect subtle changes in antigenic determinants on the receptor from different tissues and species (Kull et al., 1982; Roth et al., 1982, 1985; Ganguly et al., 1985). Similarly, they can reveal relationships betweeen the antigenic structures of the insulin and IGF-I receptors (Roth et al., 1983c), and monoclonal antibodies which specifically inhibit the binding of insulin can be used to study the role of the receptor in the actions of insulin and IGF-I (Verspohl et al., 1984). In addition, studies with one of these monoclonal antibodies have shown that receptor down-regulation can occur in the absence of insulin action or receptor phosphorylation (Roth et al., 1983b).

Clearly, monoclonal antibodies are immensely valuable tools. However, the few monoclonal antibodies available prior to the present studies have some limitations: they are directed to the external surface of the receptor and are largely human-specific. As a result,

they cannot be used in studies of the receptor cytoplasmic domain (i.e. the kinase domain) and do not recognize the receptor from the many non-human cell types used routinely in the study of insulin action.

Monoclonal antibodies as probes of receptor kinase structure and function

The underlying purpose of the studies presented in this dissertation was to use monoclonal antibodies to probe the structure and function of the insulin receptor kinase. Since, as mentioned above, no antibodies to this region were available, it was first necessary to develop a panel of monoclonal antibodies to the kinase domain. Furthermore, antibodies were needed which would recognize the kinase domain of receptors from non-human species. Thus, the work described in this dissertation begins with the development of these antibodies and then continues with the use of these antibodies in studies of the receptor kinase and its role in insulin action.

The results of these studies are presented in five chapters. Since each chapter has been written in manuscript form for publication, each contains its own summary, introduction, methods, results, and discussion. Briefly, the contents of these chapters can be introduced as follows. Chapter 1 describes a rapid and sensitive new method for screening hybridoma cells for the production of monoclonal anti-receptor antibodies. In Chapter 2, this method was used successfully in the development of a panel of 36 monoclonal antibodies to the human insulin receptor. Thirty four of these antibodies recognized the cytoplasmic kinase domain and also recognized the receptor from various species. Some of these antibodies were potent inhibitors of kinase activity, and one of these inhibitory antibodies was injected into frog oocytes and found to inhibit insulin-stimulated oocyte maturation. Chapter 3 describes the use of this panel of antibodies in mapping the antigenic regions of the receptor, and relates these regions to the receptor's functional domains. These studies also revealed that the highly inhibitory antibodies fully recognize the IGF-I receptor. Thus, in order to gain new insight into the relationship between the actions of insulin and IGF-I,

immunoaffinity techniques were used in Chapter 4 to purify the IGF-I receptor. This purified receptor and its kinase activity were then characterized by a variety of methods. Finally, Chapter 5 describes a series of crucial experiments demonstrating that the introduction of the inhibitory antibodies into the cytoplasm of various mammalian cell types results in an inhibition of several rapid insulin actions, supporting a role for the kinase in these actions.

CHAPTER 1

Plate Binding Assay for Monoclonal Anti-Receptor Antibodies

Reprinted from:

David O. Morgan and Richard A. Roth

Endocrinology, 1985, **116**, 1224-1226.

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The initial aim of the studies in this dissertation was to clarify the structure and function of the receptor kinase through the use of monoclonal antibodies. To accomplish this goal, it was first necessary to develop a panel of antibodies which, unlike those previously described, would bind to the kinase domain and would be useful in studies of the receptor from various species. The development of these antibodies was initially hampered by the relatively tedious and inefficient methods available for detecting anti-receptor antibodies in a large number of different hybridoma supernatants. Thus, as described in this chapter, a new screening technique was devised.

SUMMARY

A new procedure has been developed for rapid identification of monoclonal antibodies to the insulin receptor. The technique uses the ability of polyvinyl chloride plates coated with anti-mouse antibodies to specifically adsorb antibodies in hybridoma supernatants. Detection of anti-receptor antibodies is accomplished by allowing the adsorbed anti-receptor antibodies to bind radiolabeled hormone-receptor complexes. The method does not require pure receptor and can be adapted for studies of any receptor or binding protein where a labeled ligand is available. With small amounts of the insulin receptor (20-50 ng), the method could detect antibody concentrations as low as 300 pM. In summary, the assay is sensitive, rapid, and requires small amounts of impure receptor.

INTRODUCTION

The study of hormone receptor structure and function has been greatly facilitated by the development of the monoclonal antibody technique (Kohler and Milstein, 1975; Eisenbarth and Jackson, 1982; Fraser and Lindstrom, 1984). In this technique, immunized mouse lymphocytes are fused to myeloma cells to produce hybridoma cell lines secreting single

specific antibodies to antigens which have not been purified. To isolate a monoclonal antibody, a large number of different hybridomas must be screened for the production of the desired antibody. The screening procedure must be rapid, sensitive, unambiguous, and appropriate for the planned uses of the antibody. In the production of anti-receptor antibodies, several screening procedures have been used. Inhibition of ligand binding has been a successful technique (Roth et al., 1982; Fraser and Venter, 1980; Schreiber et al., 1981; Yavin et al., 1981; Simpson et al., 1983), but such methods are laborious and will only reveal antibodies to the binding site of the receptor. Screening by inhibition of other receptor functions, such as bioactivity, has similar limitations. Identification of antibodies to the cell surface can be achieved with radiolabeled or enzyme-linked second antibodies (Schreiber et al., 1981; Yavin et al., 1981), but such techniques will also reveal antibodies to cell surface proteins other than the desired receptor. Enzyme-linked and radiolabeled second antibodies are also used in various solid phase assays, such as immunoblotting (Gullick and Lindstrom, 1982; Gametchu and Harrison, 1984), solid phase RIA (Fraser and Lindstrom, 1984), or the enzyme-linked immunosorbent assay (ELISA)(Fraser and Lindstrom, 1984; Logeat et al., 1983). In these assays, nitrocellulose paper or the wells of plastic plates are first coated with purified antigen, followed by the hybridoma antibody and the labeled second antibody. Success with this technique requires a large amount of reasonably pure receptor. Finally, immunoprecipitation of labeled receptor or receptor binding activity has been used. This method involves the precipitation of antibody-antigen complexes by *Staphylococcus aureus* (Harford et al., 1982) or a second antibody (Logeat et al., 1983; Greene et al., 1980; Kull et al., 1982). Immunoprecipitation is useful for identifying antibodies to all regions of the receptor molecule, but the procedure is time-consuming and laborious. In the following report, a new screening technique for monoclonal anti-receptor antibodies is described which combines the advantages of immunoprecipitation and solid phase assays. The technique involves immunoprecipitation of specifically labeled receptor in 96-well polyvinyl chloride plates, using wells coated first

with anti-mouse antibodies and then with hybridoma antibody. The method is rapid, sensitive, unambiguous, and does not require pure receptor.

EXPERIMENTAL PROCEDURES

Materials

Affinity-purified rabbit anti-mouse Ig (γ heavy chain and κ light chain specific) and normal mouse IgG are from Cappel; 96-well polyvinyl chloride (PVC) plates with round-bottomed wells are from Dynatech; BSA, fraction V, bacitracin, phenylmethyl sulfonyl fluoride (PMSF), Triton X-100, chloramine-T, dimethyl sulfoxide (DMSO), Tween-20 (polyoxyethylene sorbitan monolaurate), and N-acetyl-D-glucosamine (NAG) are from Sigma; disuccinimidyl suberate (DSS) is from Pierce; wheat germ agglutinin sepharose (WGA-sepharose) is from Miles, and carrier-free $Na^{125}I$ is from Amersham. Purified porcine insulin (Elanco) was iodinated by a chloramine-T procedure as described (Roth et al., 1982) to a SA of 130 Ci/g. Control MOPC 104E IgM was purified as described (Roth and Koshland, 1982).

Methods

The initial characterization of the assay was accomplished with a well-characterized monoclonal antibody to the human insulin receptor, ARA 1 (Roth et al., 1982). ARA 1 was purified from ascites fluid with Protein A sepharose chromatography. ARA 2, which was recently discovered with the new assay (manuscript in preparation), is an IgM and was purified from ascites fluid with Sepharose 4B chromatography.

For the basic assay, 50 µl of affinity-purified rabbit anti-mouse Ig (40 µg/ml) in 20 mM $NaPO_4$ (pH 7.4)/150 mM NaCl (PBS) was added to each well of a 96-well PVC plate. Smaller amounts of anti-Ig gave less sensitivity. After an incubation of 2 to 4 h at 24°C, the wells were washed twice with 300 µl of ice-cold PBS containing 0.1% BSA

(PBS/BSA). Plate washing was accomplished by shaking off the liquid and adding the wash buffer with a squeeze bottle; 96 wells could be processed in 2 min. Fifty µl of hybridoma supernatant or anti-receptor antibody was then added for another 2 to 4 h at 24°C, followed by two washes. Fifty µl of labeled insulin receptor (next section) in PBS/BSA was added to each well for 2 h at 24°C or 16 h at 4°C. Wells were washed two more times and cut off for counting.

Receptor Labeling

For the final step of the assay, a variety of labeled receptor preparations have been used. Most screening assays have been done with human placental insulin receptor purified as described (Roth and Cassell, 1983) and cross-linked to ^{125}I-insulin with DSS. Typically, 1-5 µg receptor in 500 µl 50 mM Hepes pH 7.6/150 mM NaCl/0.1% Triton X-100 (HBS/Triton) was incubated with 1 X 10^7 cpm ^{125}I-insulin for 1 h at 24°C, followed by chilling to 0°C and the addition of 10 µl 27 mM DSS (in DMSO). After 15 min at 0°C, the reaction was stopped with 50 µl 1 M Tris pH 7.5 and the mixture was loaded onto a 2 ml WGA-sepharose column at 4°C. The column was washed with HBS/Triton and the receptor (approximately 1 µCi ^{125}I-insulin/µg receptor) was eluted with 0.3 M NAG in HBS/Triton.

Purified insulin receptor has also been labeled by radio-iodination. One hundred µl (2 µg) receptor in HBS/Triton was combined with 1 mCi Na^{125}I (10 µl) and 20 µl 1 mM chloramine-T and allowed to react 5 min at 24°C. ^{125}I-receptor (16 Ci/g) was then separated from other reaction components by chromatography on a Sephadex G-25 column (1 X 7 cm).

Another method has involved labeling the receptor with ^{125}I-insulin after precipitation on the plate. Anti-receptor antibody-coated wells are incubated with unlabeled insulin receptor, followed by a subsequent incubation with ^{125}I-insulin (40,000 cpm) in PBS/BSA. In this procedure, as in the procedure using cross-linked receptor, a highly

purified receptor preparation is not necessary. In some experiments, purified receptor (20-50 ng, or 50-150 fmol insulin binding activity) was used. In other work, partially purified insulin receptor was obtained from solubilized human placenta. In a typical preparation, 50 g of human placenta was homogenized (Brinkmann Polytron) in 100 ml 50 mM Hepes pH 7.6/1% Triton X-100/1 mM PMSF/1 mg/ml bacitracin and incubated 30 min at 24°C. The lysate was then centrifuged at 1000xg for 10 min (4°C) and then 100,000xg for 60 min (4°C). The supernatant was loaded onto a WGA-sepharose column at 4°C, the column was washed with HBS/Triton/1 mM PMSF, and the receptor was eluted with 0.3 M NAG in HBS/Triton/1 mM PMSF. Fifty µl of this eluate was used per well. For the subsequent incubation with ^{125}I-insulin, non-specific binding of insulin to the plate could be greatly reduced by the inclusion of 0.1% Tween-20 in the PBS/BSA.

RESULTS

The sensitivity of the assay was first characterized with ARA 1 as the anti-receptor antibody (Figure 1.1). Half-maximal precipitation of ^{125}I-insulin receptor (cross-linked), ^{125}I-receptor, and unlabeled purified receptor plus ^{125}I-insulin occurred at an antibody concentration of about 4 X 10^{-9} M (0.6 µg IgG/ml) (Figure 1.1). When only partially purified receptor plus ^{125}I-insulin were used (Figure 1.1, bottom), half-maximal precipitation also occurred at about 4 X 10^{-9} M.

A second monoclonal antibody to the insulin receptor, ARA 2, was found to behave quite differently in the precipitation of labeled receptor (Figure 1.2). To begin with, ARA 2 did not precipitate ^{125}I-receptor (Figure 1.2, top). However, in the precipitation of ^{125}I-insulin receptor (cross-linked) and unlabeled purified receptor plus ^{125}I-insulin, ARA 2 provided half-maximal precipitation at only 3 X 10^{-10} M (0.3 µg IgM/ml). In addition, maximum amount of receptor precipitated by ARA 2 was more than 6-fold greater than the amount precipitated by ARA 1 under identical conditions (Figure 1.1). Partially purified

receptor was also precipitated by ARA 2 with high sensitivity and capacity: half-maximal precipitation occurred at about 8 X 10^{-10} M (Figure 1.2, bottom). For unknown reasons, there was consistently lower precipitation at the highest concentrations of ARA 2. ARA 2 did not precipitate ^{125}I-insulin in the absence of the receptor.

DISCUSSION

This report describes a new procedure for identifying monoclonal antibodies to the insulin receptor. This assay utilizes the ability of anti-mouse Ig-coated microtiter wells to precipitate antibody-antigen complexes. The assay worked well with either highly purified receptor or receptor purified only by WGA-sepharose chromatography. The receptor could be labeled either by iodinating it directly or by utilizing its ability to specifically bind ^{125}I-insulin. Thus, this assay is quite versatile and may therefore be applicable to other receptors. In the study of receptors which cannot be solubilized with retention of binding activity, it may be possible to cross-link the labeled ligand to plasma membranes and then use the solubilized complex.

The assay is sufficiently sensitive to detect the amounts of antibody present in hybridoma supernatants (about 10 μg/ml) (Eisenbarth and Jackson, 1982). This level of antibody is enough to produce maximal precipitation of labeled receptor, judging by the results with either ARA 1 or ARA 2. Furthermore, this assay was used to screen 200 hybridoma supernatants. One positive supernatant was found (ARA 2), and this antibody has subsequently been shown to be directed against the receptor, since it immunoprecipitates only the receptor from metabolically labeled cells (manuscript in preparation). In addition, no false positives were found in this screening.

The differences in behavior between ARA 1 (an IgG) and ARA 2 (an IgM) are instructive. Much of the difference may simply be the result of differences in affinity for the antigen and also due to the higher avidity of the IgM molecule. ARA 1 is known to

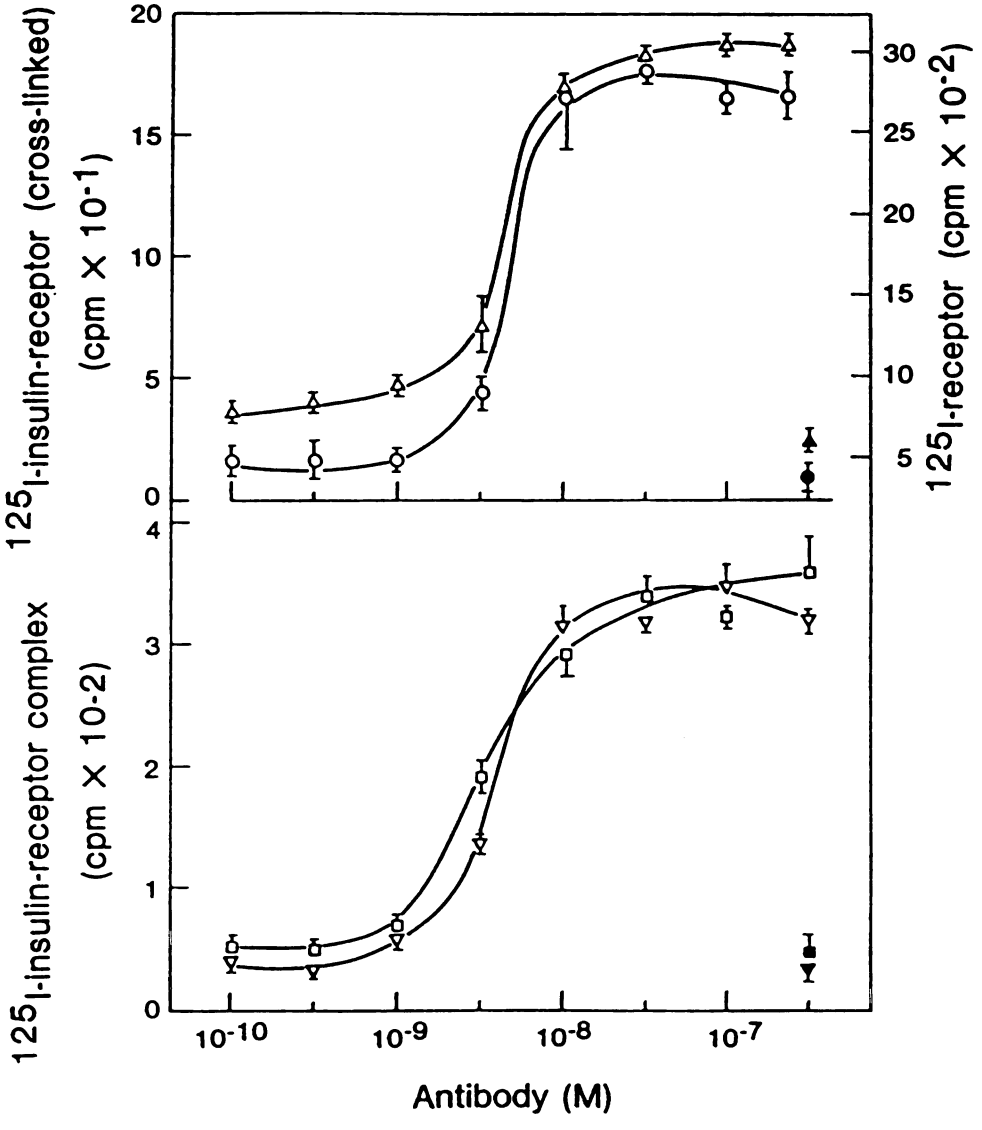
recognize the insulin binding site of the receptor (Roth et al., 1982), and so the precipitation by ARA 1 of ^{125}I-insulin-labeled receptor supports the presence of two binding sites on the receptor. The lower apparent affinity and capacity of ARA 1 for the ^{125}I-insulin-bound receptor may be partly due to the reduced number of antigenic sites on the occupied receptor. Occupation of the binding site will not hinder the binding of ARA 2 to the receptor, since this antibody does not recognize the binding site (manuscript in preparation). This illustrates one of the limitations of all assays in which the receptor is labeled by the binding of radioactive ligand: antibodies to the binding site will not be detected if the receptor under study has only one binding site. If such an antibody is desired, then iodinated pure receptor might be utilized, or the hybridomas might be screened for the production of antibodies which inhibit ligand binding. Screening by immunoprecipitation of an occupied receptor may also identify antibodies to the radioligand. Thus, the positive identification of anti-receptor antibodies requires additional assays demonstating that unoccupied receptor (^{125}I-receptor or unlabeled receptor followed by radioligand) can also be immunoprecipitated. Similarly, additional assays should demonstrate that the antibody does not precipitate the radioligand alone.

In this report, results with iodinated receptor were not as promising as those obtained with other receptor preparations. Apparently, the iodination procedure damaged antigenic sites on the receptor so that the binding of ARA 2 was abolished. More mild iodination procedures may solve this problem.

In summary, the present studies describe a technique for identifying monoclonal anti-receptor antibodies which is considerably more sensitive and less tedious than other immunoprecipitation procedures. In contrast to other solid phase assays, such as the ELISA, the present assay requires small amounts of relatively impure receptor. Moreover, it is theoretically adaptable for use with a wide range of receptors.

Figure 1.1 (next page). Plate immunoprecipitation of various insulin receptor preparations by ARA 1.

Wells were incubated with 2 μg rabbit anti-mouse IgG in PBS for 3 h at 24°C, and washed twice with PBS/BSA. Fifty μl of ARA 1 (open symbols) or normal mouse IgG (closed symbols) in PBS/BSA was added for 3 h at 24°C. Wells were washed twice, and then incubated with one of four receptor preparations, as follows. Top panel represents results with either 7000 cpm/well of ^{125}I-insulin-receptor (cross-linked) in PBS/BSA (○) or 27,000 cpm/well of ^{125}I-receptor in PBS/BSA/0.1% Tween-20 (△). Wells were incubated with labeled receptor (50 μl) for 16 h at 4°C, washed twice, and counted. Bottom panel represents results with unlabeled receptor preparations: wells were incubated for 16 h at 4°C with either purified receptor (30 ng in PBS/BSA) (□) or WGA-purified receptor (50 μl, see Experimental Procedures) (▽). Wells were washed twice, incubated 90 min at 24°C with ^{125}I-insulin (40,000 cpm) in PBS/BSA/0.1% Tween-20, washed twice, and counted. Each point represents the mean ± S.E. of triplicate wells; similar results were obtained in two separate experiments.


125I-insulin-receptor (cross-linked)
(cpm X 10-1)
125I-receptor (cpm X 10-2)
125I-insulin-receptor complex
(cpm X 10-2)
Antibody (M)
10-10
10-9
10-8
10-7

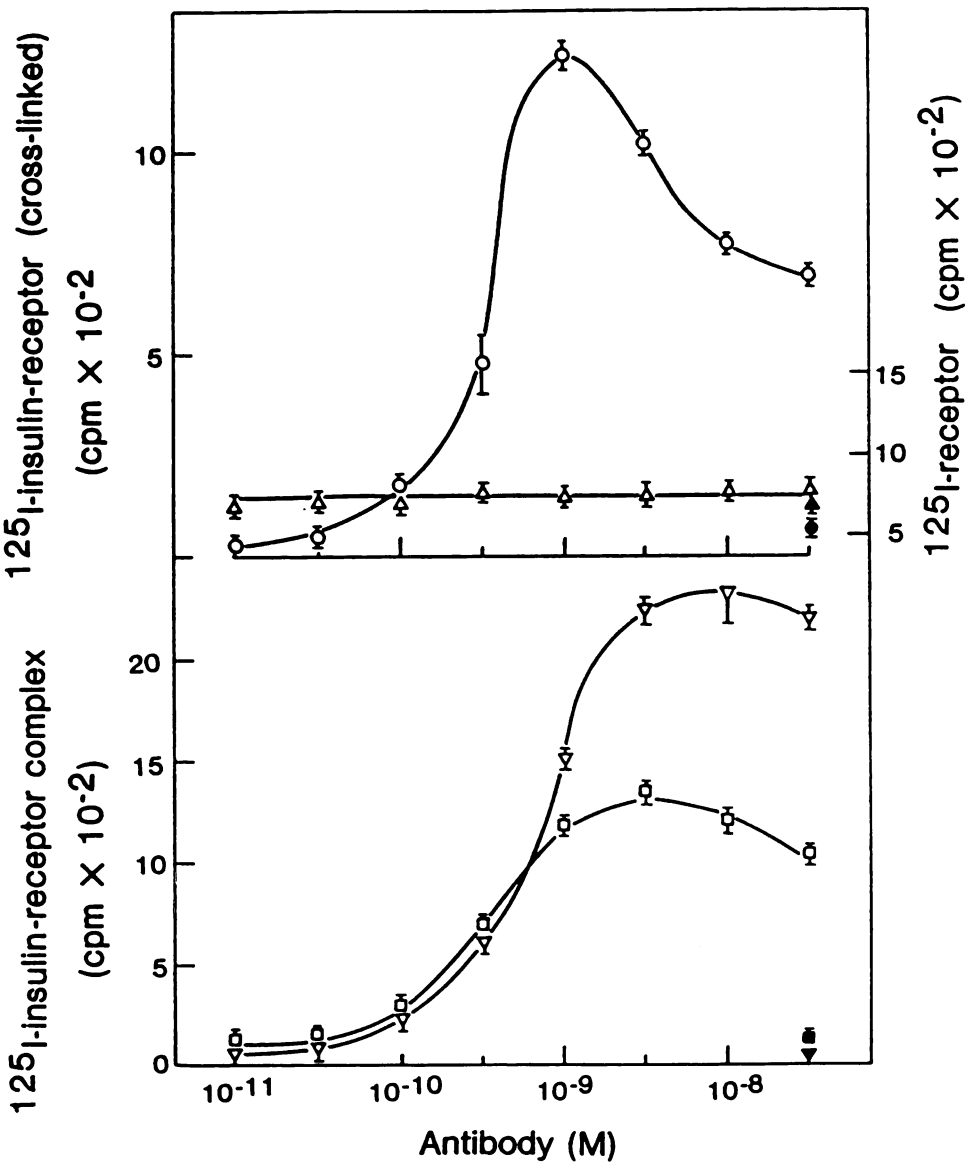


Figure 1.2. Plate immunoprecipitation of various insulin receptor preparations by ARA 2.

Procedure and symbols are identical to those in Figure 1.1, except for the substitution of ARA 2 and control IgM in this figure for ARA 1 and control IgG. Note differences in scales between Figures 1.1 and 1.2. Each point represents the mean ± S.E. of triplicate wells; similar results were obtained in two separate experiments.

CHAPTER 2

Insulin Action is Blocked by a Monoclonal Antibody that Inhibits the Insulin Receptor Kinase

Reprinted from:

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Proc. Natl. Acad. Sci. USA, 1986, **83**, 328-332.

The availability of a useful new hybridoma screening technique made it possible to continue with the first aim of this work: to develop monoclonal antibodies to the insulin receptor kinase. This chapter documents the development of a panel of antibodies to the insulin receptor, many of which were found to recognize the cytoplasmic domain and inhibit the kinase activity of insulin receptors from various species. It was also possible in this chapter to begin to use the antibodies for the study of receptor kinase structure and function. Thus, injecting frog oocytes with highly inhibitory antibodies was found to inhibit insulin-stimulated oocyte maturation, demonstrating a role for the kinase in this long-term insulin effect.

SUMMARY

Thirty-six monoclonal antibodies to the human insulin receptor were produced. Thirty-four bound the intracellular domain of the receptor β subunit, the domain containing the tyrosine-specific kinase activity. Of these 34 antibodies, 33 recognized the rat receptor and 1 was shown to precipitate the receptors from mice, chickens, and frogs with high affinity. Another of the antibodies inhibited the kinase activities of the human and frog receptors with equal potencies. This antibody inhibited the kinase activities of these receptors by more than 90%, whereas others had no effect on either kinase activity. Microinjection of the inhibiting antibody into *Xenopus* oocytes blocked the ability of insulin to stimulate oocyte maturation. In contrast, this inhibiting antibody did not block the ability of progesterone to stimulate the same response. Furthermore, control immunoglobulin and a noninhibiting antibody to the receptor β subunit did not block this response to insulin. These results strongly support a role for the tyrosine-specific kinase activity of the insulin receptor in mediating this biological effect of insulin.

INTRODUCTION

Insulin initiates its diverse biological effects by binding to its receptor, an integral membrane glycoprotein composed of two α (M_r=135,000) and two β (M_r=95,000) subunits linked by disulfide bonds (Kahn, 1983). Recently, the insulin receptor, like the receptors for several other growth factors and various viral oncogene proteins, has been shown to have an intrinsic tyrosine-specific protein kinase activity (Heldin and Westermark, 1984). Since many of the effects of insulin are caused by changes in the phosphorylation state of various proteins (Avruch et al., 1982), it has been suggested that some or all of the effects of insulin are mediated by the tyrosine-specific kinase activity of its receptor. There are several lines of evidence which support this hypothesis: (i) these is reduced kinase activity of the receptor kinase in cells from certain patients with diabetic syndromes characterized by lack of responsiveness to insulin (Grunberger et al., 1984; Grigorescu et al., 1984); (ii) phorbol diesters cause a parallel decrease in insulin responsiveness of cells and insulin-stimulated tyrosine kinase activity of the receptor (Takayama et al., 1984); and (iii) various agents (i.e., lectins, trypsin, or vanadate) which mimic the effects of insulin on intact cells also stimulate the tyrosine kinase activity of the receptor (Roth et al., 1983a; Tamura et al., 1983, 1984). Evidence against the insulin receptor kinase mediating the biological effects of insulin arises principally from studies utilizing various polyclonal anti-receptor antibodies. Two of these antisera have been shown to mimic the ability of insulin to stimulate glucose uptake in rat adipocytes without stimulating either the kinase activity of solubilized receptor preparations or increasing the extent of receptor phosphorylation in the intact adipocyte (Simpson and Hedo, 1984; Zick et al., 1984a).

To further define the role of the receptor kinase in mediating the biological effects of insulin, we set out to produce monoclonal antibodies that inhibit this activity and then to determine whether microinjection of these antibodies into cells would affect the ability of

the cells to respond to insulin. Although several monoclonal antibodies to the human insulin receptor have previously been described (Roth et al., 1982; Kull et al., 1983), all of these antibodies are directed to the extracellular domain of the receptor and are human-specific. In the present report we describe the production of monoclonal antibodies to the intracellular domain of the human receptor, several of which inhibit the receptor kinase and recognize the insulin receptors of a variety of species. These antibodies were microinjected into *Xenopus* oocytes and examined for their effect on the ability of insulin to stimulate oocyte maturation, a known effect of insulin (El-Etr et al., 1979; Maller and Koontz, 1981). Moreover, since these cells have a distinct receptor for progesterone (Sadler and Maller, 1982) and progesterone also stimulates oocyte maturation (Reynhout et al., 1975), the effect of the antibodies on this response was also examined.

EXPERIMENTAL PROCEDURES

Insulin Receptor Purification

Insulin receptors from human placenta, rat liver, and *Xenopus* oocytes were highly purified by sequential affinity chromatography on an anti-receptor antibody column and wheat germ agglutinin column as described (Roth and Cassell, 1983) except that the anti-receptor affinity column was composed of 10 of the monoclonal anti-receptor antibodies described in this work (3D7, 11B11, 15B5, 17A3, 17E5, 17H5, 20G2, 24B7, 27B2, and 28B7). Labeled receptor was prepared by covalently cross-linking ^{125}I-labeled insulin (^{125}I-insulin) to purified placental receptor with disuccinimidyl suberate as described (Morgan and Roth, 1985). In some experiments (see Figure 2.2), insulin receptors from human placenta, mouse hepatoma cells (Hepa 1), and chicken liver were partially purified by wheat germ lectin affinity chromatography and then were labeled by covalent cross-linking to ^{125}I-insulin with disuccinimidyl suberate.

Antibody Production

Splenic lymphocytes from mice immunized with purified human placental insulin receptor were fused with Sp 2/0 myeloma cells (Shulman et al., 1978) by a modification (De St. Groth and Scheidegger, 1980) of the procedure originally described by Kohler and Milstein (1975). Supernatants from the resulting hybridomas were screened for anti-receptor antibodies with a plate immunoprecipitation assay as described (Morgan and Roth, 1985). In brief, hybridoma supernatants were incubated in microtiter wells previously coated with anti-mouse IgG (40 μg/ml). After 2 h at 24°C, the wells were washed twice, and 50 μl of ^{125}I-insulin crosslinked to purified placental receptor (10,000 cpm) was then added. After an additional 2 h incubation, the wells were washed two more times and cut off for assay. Positive hybridoma supernatants had 5-20 times as much radioactivity in cpm as did negative hybridoma supernatants. In the first fusion with an immunized BALB/c mouse, 1 positive hybridoma was detected in 200 cultures; in the second fusion with an immunized SJL mouse, 43 positive hybridomas were detected in 446 cultures. Of these 44 hybridomas, 36 were successfully grown up with retention of antibody production.

Cell Surface Labeling

IM-9 lymphoid cells (2 X 10^6 cells) were incubated 1 h at 15°C with 300 μl of hybridoma supernatant, followed by one wash with cold buffer (20 mM Tris-HCl, pH 7.4/ 150 mM NaCl). Cells were then resuspended in culture medium containing ^{125}I-labeled rabbit anti-mouse IgG (40,000 cpm), incubated 90 min at 15°C, washed twice with cold buffer, and assayed for radioactivity.

Plate Precipitation of Rat Receptor and Immunoprecipitation of Metabolically Labeled Cell Lysates

Microtiter wells were coated with anti-mouse IgG and hybridoma supernatants as described above and then incubated with 250 ng of purified rat liver receptor. After 16 h at 4°C, the

wells were washed twice, incubated 90 min at 24°C with ^{125}I-insulin (48,000 cpm), washed again, and assayed for radioactivity. Rat hepatoma cells (H4 cells) were labeled with [^{35}S]methionine, solubilized, and partially purified by wheat germ lectin affinity chromatography as described (Roth and Cassell, 1983). Twenty microliters of protein A-Sepharose coated with anti-mouse IgG was incubated 2 h at 24°C with 20 μg of antibody 7D5 or normal mouse immunoglobulin. After two more washes, the pellet was resuspended in the labeled cell lysate and incubated 16 h at 4°C. After 4 washes, the precipitated proteins were subjected to $NaDodSO_4$/poly-acrylamide gel electrophoresis and autoradiography.

Effect of Antibodies on Receptor Kinase Activity

Purified insulin receptor from human placenta or *Xenopus* oocytes (≈25 ng) was incubated 1 h at 24°C in a 15 μl reaction mixture containing 50 mM Hepes (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 2 mM $MnCl_2$, 1 mM insulin, 0.5 mg of bovine serum albumin per ml, and the desired concentration of protein A-purified monoclonal antibody. [γ-^{32}P]ATP (ICN, 23 Ci/mmol; 1 Ci=37 GBq) was then added (final concentratioon, 10 μM) with or without 5 μg of histone H2b; after 1 h at 24°C, reaction mixtures were analyzed by $NaDodSO_4$ gel electrophoresis. The 95-kDa β-subunit band and the histone band were excised and assayed in a liquid scintillation counter.

Oocytes

Stage VI oocytes (Dumont, 1972) from female *Xenopus laevis* (Nasco, Fort Atkinson, WI) primed with human chorionic gonadotropin (Sigma) were manually dissected from their ovarian follicle, placed in Barth's medium (10 mM Tris-HCl/8.8 mM NaCl/2.4 mM $NaHCO_3$/0.8 mM $MgSO_4$/1 mM $CaCl_2$/10 mM Hepes, pH 7.6), and microinjected into the cytoplasm with the desired antibody (40 nl per oocyte). Injected oocytes were incubated 1 h at 19°C, and healthy oocytes (20-25) were then transferred to Barth's medium (10

oocytes per 1 ml) containing 0.1% bovine serum albumin and the desired hormone. After 18 h at 19°C, oocytes were analyzed for maturation by the appearance of a "white spot" in the pigmented animal pole. This previously has been shown to correlate with induction of germinal vesicle breakdown (Maller and Koontz, 1981) and was confirmed in the present studies.

RESULTS

Characterization of the Monoclonal Anti-Receptor Antibodies

Thirty-six hybridomas producing anti-receptor antibodies were identified in two fusions by their ability to precipitate ^{125}I-insulin receptor complexes (Table 2.1) and iodinated receptor (not shown) in a plate precipitation assay (Morgan and Roth, 1985). Only 2 of the 36 antibodies (3D7 and 5D9) were found to label the outside surface of intact human IM-9 lymphoid cells to the same extent as a previously described monoclonal antibody (MC51) to an extracellular site on the receptor (Table 2.1). This result suggested that the remaining 34 antibodies recognize an intracellular or transmembrane site on the receptor. Since previous work (Ullrich et al., 1985; Ebina et al., 1985b; Hedo and Simpson, 1984) has indicated that only the β subunit has a transmembrane sequence and is exposed to the intracellular environment, these results suggested that these 34 antibodies were directed against the β subunit.

The contribution of the β subunit to the sites recognized by these antibodies was further examined by utilizing the ability of crude collagenase preparations to extensively degrade the β subunit without affecting the α subunit (Roth et al., 1983d). Collagenase digestion was found not to affect the precipitation of the receptor by the 2 antibodies to the extracellular portion of the receptor, whereas precipitation by the remaining 34 antibodies was inhibited by 80% (Table 2.1). When various concentrations of collagenase were utilized, the loss in immunoprecipitation by one of these antibodies (7D5) exactly paralleled

the proteolysis of the β subunit (Figure 2.1). In contrast, proteolysis had no effect on either the integrity of the α subunit or the precipitation of the receptor with one of the antibodies to the extracellular domain (MC51) (Figure 2.1). These results with the proteolyzed receptor support the hypothesis that 34 of the antibodies are directed against the β subunit.

Species Specificity of the Monoclonal Antibodies

Since prior studies with two monoclonal antibodies to extracellular sites on the human receptor suggested that these antibodies were species-specific (Roth et al., 1982; Kull et al., 1983), all 36 antibodies were tested for their ability to recognize the rat insulin receptor. The 2 antibodies that recognize extracellular sites on the receptor (3D7 and 5D9) did not precipitate the receptor from rat liver (Table 2.1). In contrast, 33 of the 34 antibodies to the intracellular domain of the β subunit did precipitate the rat receptor (Table 2.1). Additional studies with one of these antibodies (7D5) indicated that this antibody could precipitate the human, mouse, chicken, and frog receptors with almost equal affinity (Figure 2.2). Immunoprecipitation of metabolically labeled lysates from rat hepatoma cells with this antibody (7D5) confirmed that this antibody recognized the rat receptor and, furthermore, demonstrated that this antibody did not cross-react with other cellular proteins (Figure 2.2 *Inset*).

Effect of the Antibodies on the Receptor Kinase

Nine of the antibodies were then examined for their ability to interfere with the receptor kinase. The effects of the different antibodies varied: autophosphorylation and phosphorylation of the exogenous substrate (histone) by the human placental receptor was inhibited from 99% to 10% (Figure 2.3). The effects of these antibodies on the kinase activity of the frog oocyte receptor paralleled those found with the human receptor, although some small differences were noted (Figure 2.3). The effect of one of the highly inhibitory antibodies (17A3) on the frog oocyte and the human placental receptor kinases

was examined in more detail (Figure 2.4). Both autophosphorylation and exogenous substrate phosphorylation by the frog oocyte receptor were inhibited half-maximally by this antibody (17A3) at 5 nM. The pattern of inhibition of the human placental receptor kinase by this antibody was almost identical (Figure 2.4). In contrast, another antibody (1G2) at 300 nM had no effect on either autophosphorylation or phosphorylation of histone by either receptor (Figure 2.4).

Microinjection Experiments

Since several of the monoclonal antibodies to the intracellular domain of the β subunit inhibited the kinase activity of the frog oocyte insulin receptor, it was possible to directly test the role of the receptor kinase by microinjecting these antibodies into oocytes and examining their effect on the ability of insulin to stimulate maturation. In one such study, in the absence of insulin, none of the oocytes initiated germinal vesicle breakdown after 19 h. In contrast, in the presence of 1 mM insulin, 62% of the oocytes were positive for germinal vesicle breakdown. Oocytes that were injected with the inhibiting antibody 17A3 showed a greatly decreased response to insulin. When oocytes were injected with sufficient antibody 17A3 to yield an intracellular concentration of 13 nM and treated with 1 μM insulin, only 9% were positive for germinal vesicle breakdown (Table 2.2), an inhibition of the insulin response by 80%. In two additional experiments, the inhibition observed with 13 nM antibody 17A3 was 79% and 90%. This inhibition was dose-dependent; 1.3 and 0.13 nM antibody 17A3 inhibited the response to insulin by 42% and 10%, respectively (Table 2.2). In contrast to these results with the inhibiting antibody, no inhibition was observed by the injection of either 13 nM control mouse immunoglobulin or 13 nM of the noninhibiting antibody 1G2 (Table 2.2).

One explanation for the inhibition of the response to insulin after microinjection of antibody 17A3 would be that insulin binding was decreased as a result of antibody-induced receptor clustering and internalization. However, oocytes microinjected with the different

antibodies and incubated for 18-20 h all bound the same amount of ^{125}I-insulin (data not shown).

Since progesterone also can stimulate maturation of oocytes (Reynhout et al., 1975), the specificity of the inhibiting antibodies could be tested by examining their effect on this response. Progesterone at 1 μM stimulated 96% of the oocytes to germinal vesicle breakdown. Microinjection of inhibiting antibody 17A3, noninhibiting antibody 1G2, or control immunoglobulin to yield a concentration of 13 nM resulted in 77%, 80%. and 74%, respectively, of the oocytes initiating germinal vesicle breakdown, values that were not significantly different (Table 2.2). The lack of effect of the inhibiting antibody 17A3 on the progesterone response was demonstrated in two additional experiments.

DISCUSSION

In this report we describe the generation of a panel of 36 monoclonal antibodies to the human insulin receptor. Thirty-four of these antibodies are most likely directed against the intracellular domain of the receptor β subunit, since they do not react with the extracellular domain of the receptor (Table 2.1). Also, proteolysis of the β subunit inhibits the ability of these same 34 antibodies to bind to the receptor (Table 2.1 and Figure 2.1). In addition, several of these antibodies bind to a genetically engineered truncated form of the receptor containing only the intracellular domain of the β subunit (L. Ellis, D.O.M., R.A.R., and W. Rutter, unpublished data). However, these 34 antibodies do not all bind to the same antigenic site on the receptor, since they vary considerably in their ability to inhibit the receptor kinase (Figure 2.3).

Interestingly, the intracellular domain of the receptor β subunit also appeared to be highly conserved. Thirty-three of the 34 monoclonal antibodies to this domain were found to cross-react with the insulin receptor from rat liver (Table 2.1). Furthermore, 1 of these antibodies (7D5) precipitated the human, mouse, chicken, and frog receptors with almost

equal high affinity (Figure 2.2). Finally, 1 of these antibodies (17A3) was found to inhibit the frog oocyte receptor kinase with the same potency that it inhibits the human placental receptor kinase activity (Figure 2.4). In contrast, none of the antibodies to the extracellular domain recognized the rat liver insulin receptor (Table 2.1). This may indicate that antigenic sites in the extracellular domain are not as well conserved as the intracellular sites, perhaps due to differences in glycosylation. Alternatively, the antibodies to the extracellular domain that crossreact with the receptors of other species may be selected against when making hybridomas, since such antibodies might be toxic to the mouse hybridomas that possess insulin receptors and require insulin for growth (Chang et al., 1980).

Although the monoclonal antibodies to the intracellular domain of the β subunit react with the insulin receptors from a variety of species, they are highly specific for the insulin receptor. For example, antibody 7D5 only precipitated the insulin receptor from metabolically labeled cells (Figure 2.2). In addition (unpublished studies), these antibodies did not recognize several other tyrosine-specific protein kinases, including the receptors for epidermal growth factor and platelet-derived growth factor, as well as the protein products of the viral oncogenes v-*fms* and v-*ros* (Heldin and Westermark, 1984; Feldman et al., 1982). These results indicate that the insulin receptor kinase is not closely related to the other tyrosine kinases, despite the limited homologies in amino acid sequence (Ullrich et al., 1985; Ebina et al., 1985b).

The ability of some of these antibodies to inhibit the receptor kinase of frog oocytes allowed us to use these antibodies to test directly the role of the receptor kinase in mediating insulin action. Microinjection of the inhibiting antibody 17A3 into frog oocytes was found to block the ability of insulin to stimulate oocyte maturation (Table 2.2). Several lines of evidence indicate that the inhibitory effect of this antibody is specific. First, the microinjection of the inhibiting antibody 17A3 had no effect on the stimulation of maturation by progesterone, another hormone that induces maturation by binding to its own distinct receptor (Sadler and Maller, 1982). Second, the dose-response curve for the

inhibition of insulin-induced maturation by 17A3 was almost the same as for the inhibition of the kinase activity of the purified receptor by this antibody (Table 2.2 and Figure 2.4). Finally, microinjection of an antibody (1G2) to the intracellular domain of the insulin receptor, which does not inhibit the kinase activity of purified receptor (Figure 2.4), had no effect on the ability of the oocytes to respond to insulin (Table 2.2).

The importance of tyrosine kinases in initiating maturation of oocytes has been suggested by the finding that microinjection of the $pp60^{v\text{-}src}$ tyrosine kinase into oocytes accelerates the rate of progesterone-induced maturation (Spivack et al., 1984). The present studies extend these conclusions to indicate that the intrinsic kinase activity of the oocyte insulin receptor is important in mediating this response of the oocyte to insulin. Additional studies with other cell types and insulin responses will be required to determine whether all of the effects of insulin require activation of the receptor kinase. For example, it may be that the more rapid effects of insulin, such as the stimulation of glucose uptake, do not require an activation of the receptor kinase. In addition, it may be that the inhibiting monoclonal antibodies have effects on the insulin receptor other than inhibiting its kinase activity. Thus, it will be important to confirm these results with other methods, such as the expression of specific mutants of the receptor in transfected cells.

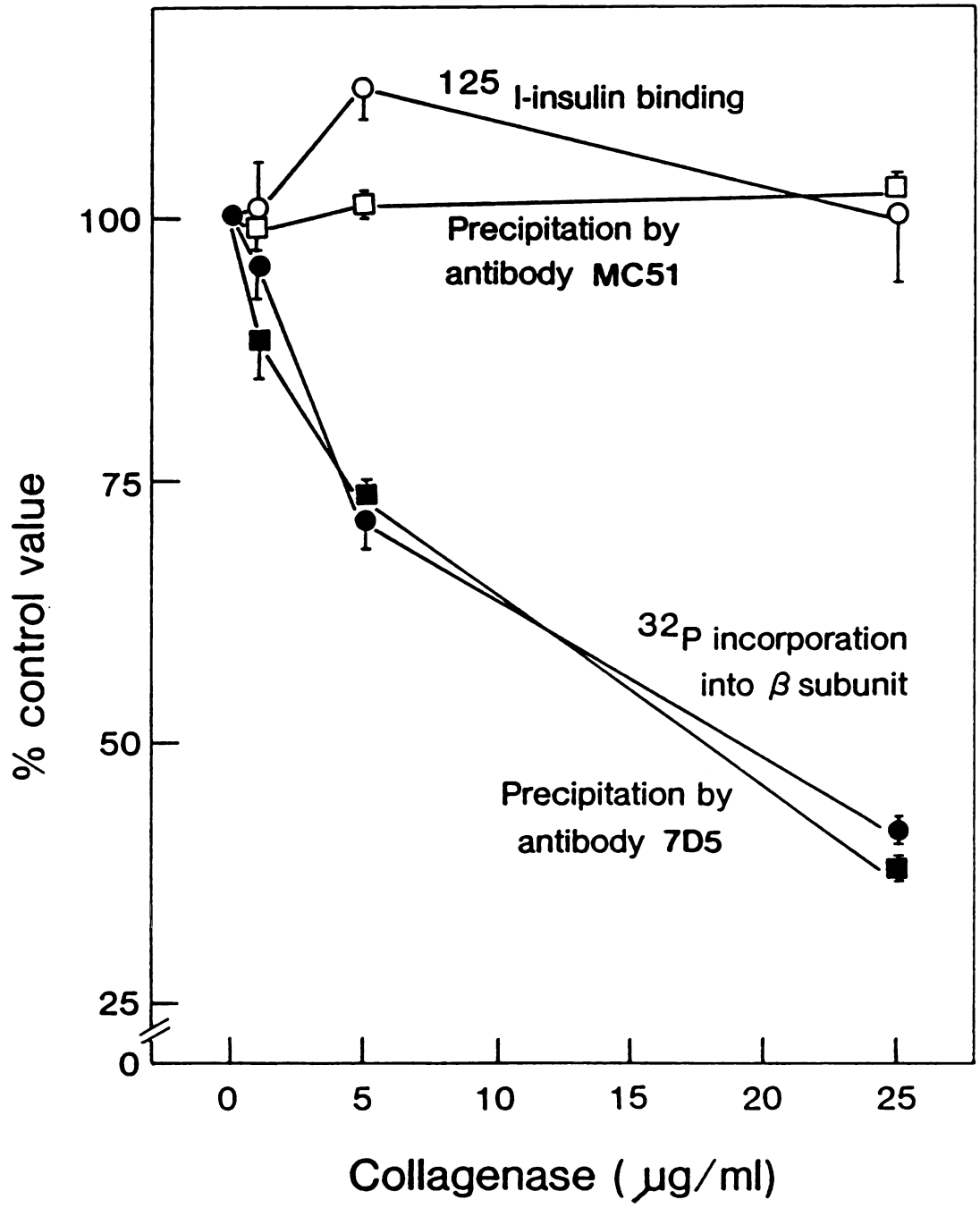
Table 2.1. Characteristics of monoclonal antibodies to the insulin receptor.

Antibody	Cell surface binding (cpm)*	Inhibition of receptor precipitation by β-subunit proteolysis (%)†	Precipitation of rat receptor (cpm)#
MC51**	6663	0	41
3D7	6500	0	156
5D9	10733	0	120
1C1	1038	82	855
1G2	717	78	978
2G7	764	69	3860
3F10	944	80	1692
4E10	725	81	1870
7D5	995	79	2243
8H2	850	81	1161
11B11	609	81	1306
13B4	837	81	1272
15B5	862	78	2858
16E8	675	75	2752
16F5	733	81	1317
17A3	748	79	2370
17E5	857	78	2428
17G6	690	81	782
17H5	692	82	414
19E3	646	83	427
19H9	625	82	1077
20B4	555	83	622
20D9	885	80	2457
20G2	895	78	2781
21C11	683	83	1492
21D3	813	80	2898
24B7	608	79	2503
24D5	944	73	626
25D4	831	83	1267
25D8	923	78	315
27B2	636	82	1454
28B7	740	80	2732
28F2	740	79	1885
29B4	997	78	2723
29E3	685	71	103
30D1	587	80	634
30D3	908	82	1319

*Non-specific labeling, determined by incubating cells with normal mouse Ig, was 764 cpm. Only values that were more than twice this value were considered positive.
†Percentage decrease in receptor binding after proteolysis, relative to binding of untreated receptor (see the legend for Figure 2.1 for experimental details).
#Nonspecific binding in the absence of mouse antibody has been subtracted (190 cpm).
**MC51 is the previously described monoclonal antibody to the insulin receptor binding site (Roth et al., 1982).

Figure 2.1 (next page). Effect of β-subunit proteolysis on receptor function and antibody recognition.

Each reaction mixture contained ≈250 ng of highly purified human placental insulin receptor in 5 μl (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% Triton X-100). To monitor the integrity of the β subunit (●), receptor was autophosphorylated, collagenase-digested (Roth et al., 1983d), and electrophoresed on reduced NaDodSO4/polyacrylamide gels. The 95 kDa β-subunit phosphorylated band was cut out and assayed in a liquid scintillation counter. To monitor the integrity of the α subunit (○), collagenase digested receptor was incubated 1 h at 24°C with 150,000 cpm of ^{125}I-insulin (120 Ci/g), treated with the cross-linking reagent disuccinimidyl suberate (0.5 mM) for 15 min at 0°C, and then subjected to gel electrophoresis. The 135 kDa α-subunit band was cut out and assayed in a γ counter. Receptors in the third and fourth groups of tubes were collagenase-digested, brought to a 50 μl volume with phosphate-buffered saline containing bovine serum albumin (saline/albumin), and added to wells of a microtiter plate coated with either MC51, a monoclonal antibody to the insulin binding site (□), or monoclonal antibody 7D5 (■). After 3 h at 24°C, the wells were washed twice with saline/albumin and then incubated with 50 μl of ^{125}I-insulin (40,000 cpm) in saline/albumin for 90 min at 24°C. Wells were washed, cut off, and assayed for radioactivity. Each point represents the mean ± S.E. of triplicates. The control values (100%) in the absence of collagenase were: 19,394 cpm (●); 1812 cpm (○); 1513 cpm (□); and 1157 cpm (■).


125 I-insulin binding
Precipitation by antibody MC51
32P incorporation into β subunit
Precipitation by antibody 7D5
% control value
100
75
50
25
0
0
5
10
15
20
25
Collagenase (μg/ml)

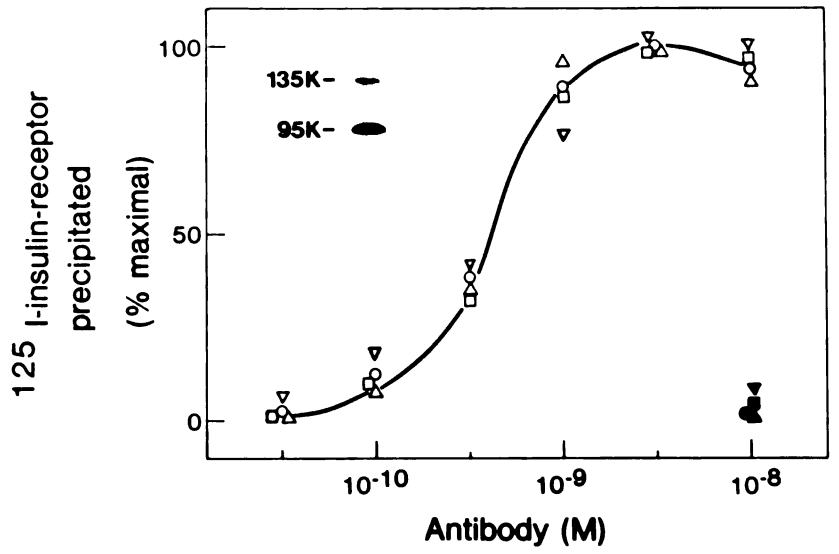


Figure 2.2. Precipitation of mammalian, avian, and amphibian insulin receptors by antibody 7D5.

Microtiter wells were coated with normal mouse immunoglobulin (closed symbols) or various concentrations of antibody 7D5 (open symbols) as described. In one series of wells, 50 µl of ^{125}I-insulin-labeled human (8100 cpm) (○), mouse (1600 cpm) (□), and chicken (8100 cpm) (△) receptor was added to antibody-coated wells, incubated for 16 h at 4°C, washed, and assayed for radioactivity. In another series, purified frog oocyte receptor (▽) was added to antibody-coated wells and incubated for 16 h at 4°C. After two washes, ^{125}I-insulin (56,000 cpm) was added for 90 min at 24°C, and the wells were washed and assayed. Maximal values (100%): ○, 1447 cpm; □, 295 cpm; △, 622 cpm; ▽, 200 cpm. (*Inset*) ^{35}S-labeled lysates of rat hepatoma cells were immunoprecipitated with antibody 7D5 as described, and the precipitate was electrophoresed: a picture of the autoradiogram is shown, with molecular weights indicated in kDa.

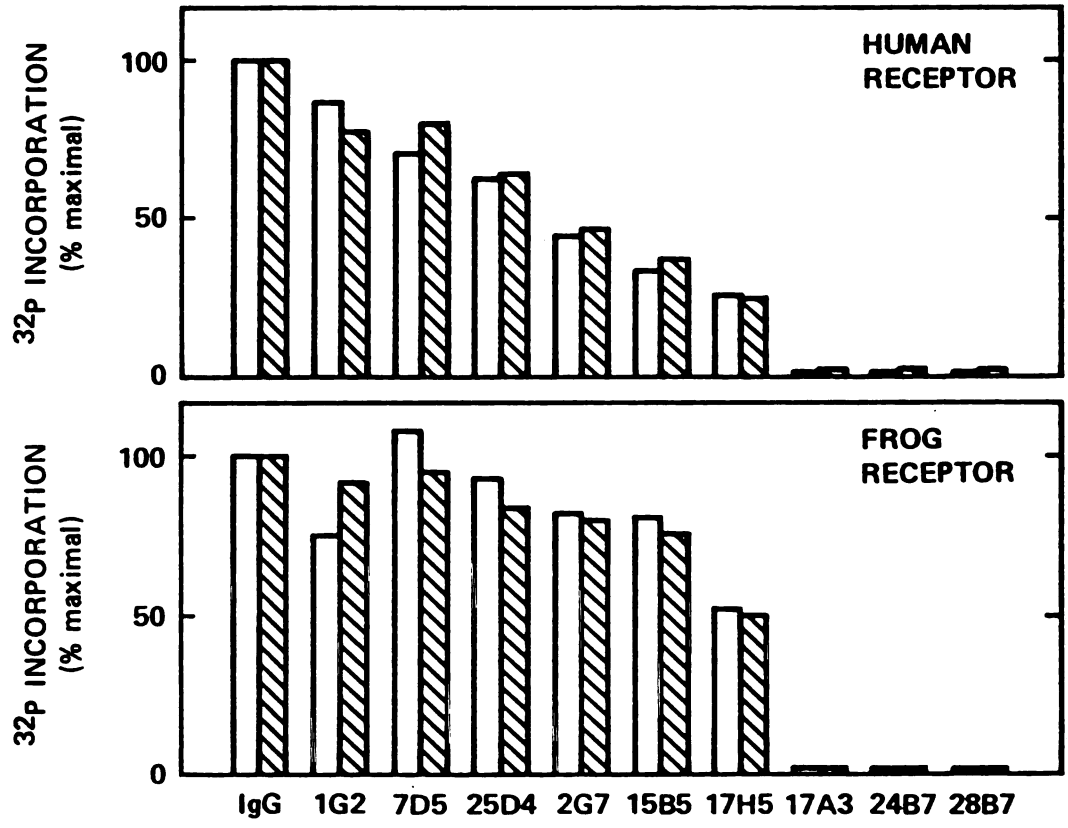


Figure 2.3. Effect of nine monoclonal antibodies on the kinase activities of purified human placental and frog oocyte insulin receptors.

Purified human placental (upper) or *Xenopus* oocyte (lower) insulin receptors were incubated with 100 nM of the indicated antibodies and tested for their ability to either autophosphorylate (open bars) or phosphorylate histone (hatched bars) as described. All values are means of duplicates.

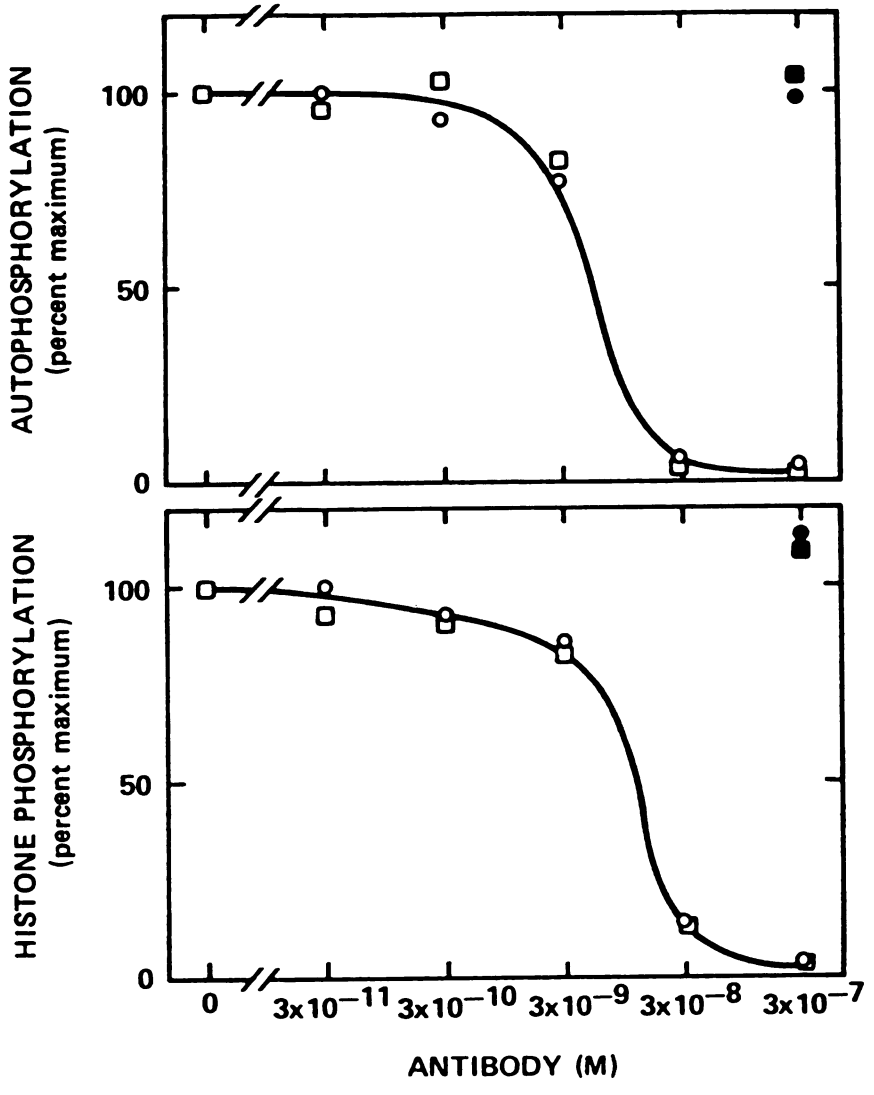


Figure 2.4. Comparison of the inhibitory potency of antibody 17A3 on the kinase activities of human and frog insulin receptors.

Purified human placental (o) or *Xenopus* oocyte (□) insulin receptors were incubated with antibody 17A3 (open symbols) or antibody 1G2 (closed symbols). Kinase acitivity was then determined by measuring ^{32}P incorporation from [γ-^{32}P]ATP into the receptor β subunit (upper) or histone (lower) as described. All values are means of duplicates.

Table 2.2. Effect of microinjection of anti-receptor antibodies on oocyte maturation.

Microinjection		Positive oocytes after hormonal treatment (%)			
Antibody	nM	No treatment	10 μM Insulin	1 μM Insulin	1 μM Progesterone
None		0	57	62	96
Mouse IgG	13	10	45	57	74
Noninhibiting anti-receptor IgG (1G2)	13	0	68	44	80
Inhibiting anti-receptor IgG (17A3)	13	10	17	9	77
	1.3	NT	NT	33	NT
	0.13	NT	NT	56	NT

Oocytes, injected with the indicated antibodies, were placed in medium containing the indicated concentrations of hormone. The numbers shown are the percentage of oocytes that were scored positive for maturation. Antibody concentrations are the final concentrations in the oocyte and were calculated by assuming an intracellular volume of 1 microliter per oocyte. NT, not tested.

CHAPTER 3

Mapping Surface Structures of the Human Insulin Receptor with Monoclonal Antibodies: Localization of the Main Immunogenic Regions to the Receptor Kinase Domain

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Biochemistry, 1986, **25**, 1364-1371.

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In the previous chapter, studies were presented which demonstrated that the antibodies to the kinase domain vary considerably in their ability to affect kinase function. Thus, it was apparent that the antibodies recognize several receptor regions, some of which are crucially involved in kinase activity. These issues are further explored in the present chapter. Competitive binding experiments were used to map the antigenic regions of the kinase domain, and relationships between these antigenic regions and receptor functional domains were then revealed by various functional assays.

SUMMARY

A panel of 37 monoclonal antibodies to the human insulin receptor has been used to characterize the receptor's major antigenic regions and their relationship to receptor functions. Three antibodies recognized extracellular surface structures, including the insulin binding site and a region not associated with insulin binding. The remaining 34 antibodies were directed against the cytoplasmic domain of the receptor β subunit. Competitive binding studies demonstrated that four antigenic regions (β_1, β_2, β_3, and β_4) are found on this domain. Sixteen of the antibodies were found to be directed against β_1, nine against β_2, seven against β_3, and two against β_4. Antibodies to all four regions inhibited the receptor-associated protein kinase activity to some extent, although antibodies directed against the β_2 region completely inhibited the kinase activity of the receptor both in the autophosphorylation reaction and in the phosphorylation of an exogenous substrate, histone. Antibodies to the β_2 region also did not recognize autophosphorylated receptor. In addition, antibodies to this same region recognized the receptor for insulin-like growth factor I (IGF-I) as well as the insulin receptor. In contrast, antibodies to other cytoplasmic regions did not recognize the IGF-I receptor as well as the insulin receptor. These results indicated that the major immunogenic regions of the insulin receptor are located on the cytoplasmic domain of the receptor β subunit and are associated with the tyrosine-specific

kinase activity of the receptor. In addition, these results suggest that a portion of the insulin receptor is highly homologous to that of the IGF-I receptor.

INTRODUCTION

Insulin initiates its diverse biological responses by binding to its receptor, an integral membrane glycoprotein composed of two α (M_r=135,000) and two β (M_r=95,000) subunits joined by disulfide bonds [for review see Kahn (1983) and Jacobs & Cuatrecasas (1981)]. Covalent cross-linking of ^{125}I-insulin to the receptor suggests that the α subunit is the major site of insulin binding (Kahn, 1983; Jacobs & Cuatrecasas, 1981; Pilch & Czech, 1979). The binding of insulin activates a tyrosine-specific protein kinase activity that is tightly associated with the receptor (Kasuga et al., 1982c; Petruzzelli et al., 1984; Van Obberghen et al., 1983; Roth &Cassell, 1983; Shia & Pilch, 1983), and several lines of evidence suggest that this kinase activity is located in the receptor β subunit. First, the β subunit contains an ATP binding site (Van Obberghen et al., 1983; Roth & Cassell, 1983; Shia & Pilch, 1983) and several autophosphorylation sites (Kasuga et al., 1982b). Second, specific proteolysis of the β subunit abolishes the kinase activity of the receptor without affecting insulin binding activity (Roth et al., 1983d; Shia et al., 1983). In addition, recent studies of the amino acid sequence of the receptor, deduced from cDNA sequences, indicate that the cytoplasmic domain of the β subunit contains sequences homologous to the ATP binding site of other kinases (Ullrich et al., 1985; Ebina et al., 1985b).

Recent studies have also clarified the spatial orientation of the receptor subunits in the cell membrane. Labeling studies indicate that both subunits are exposed to the extracellular environment, whereas only the β subunit is exposed to the cytoplasm (Hedo & Simpson, 1984). Similarly, the amino acid sequence of the receptor indicates that the α subunit is completely extracellular, whereas the β subunit contains domains on both sides of the membrane (Ullrich et al., 1985; Ebina et al., 1985b).

The immunochemical structure of the insulin receptor is not as well defined. Mapping of immunogenic regions can provide information about receptor structure, as demonstrated in the extensive studies of the acetylcholine receptor by Lindstrom and colleagues (Tzartos et al., 1981; Lindstrom et al., 1983). As yet, thorough immunochemical characterization of the insulin receptor has not been feasible. Although several monoclonal antibodies to the insulin receptor have been previously described (Roth et al., 1982; Kull et al., 1983), these antibodies have been directed to a limited number of antigenic regions, mainly on the extracellular surface of the receptor. Thus, in an attempt to clarify certain structural properties of the receptor, we have developed a panel of 37 monoclonal antibodies to the human placental insulin receptor (Morgan et al., 1986a). Thirty-four of these antibodies were found to recognize the cytoplasmic domain of the β subunit and interact with regions associated with the receptor kinase activity (Morgan et al., 1986a). The remaining three antibodies were found to bind to the extracellular domain of the receptor, including the insulin binding site (Morgan et al., 1986a). In this work, we have used these antibodies to characterize the major antigenic regions of the receptor and the relationship of these regions to the functional domains of the receptor.

EXPERIMENTAL PROCEDURES

Materials

Affinity purified rabbit anti-mouse IgG (specific for γ heavy chain and κ light chain) and affinity purified goat anti-mouse IgG (Fc fragment specific) are from Cappel; protein A is from Pharmacia; histone H2b is from Worthington; ATP and 5'-adenylyl imidodiphosphate (AMP-PNP) are from Sigma; [γ-^{32}P]ATP (23 Ci/mmol) is from ICN. Synthetic IGF-I was the generous gift of Dr. J. Merryweather of Chiron Corp. (Emeryville, CA) and was iodinated with a chloramine-T method to a specific activity of 120 Ci/g. All other materials were obtained as previously described (Roth et al., 1983d; Morgan et al., 1986a).

Insulin Receptor Purification and Labeling

Human placental insulin receptor (100 mg/placenta) was highly purified by sequential affinity chromatography on an anti-receptor antibody column and wheat-germ agglutinin column as previously described (Roth and Cassell, 1983). Coomassie-stained polyacrylamide gels of these preparations yield only the 135 kDa and 95 kDa bands of the insulin receptor (Roth & Morgan, 1985). Labeled insulin receptor was prepared by covalently cross-linking ^{125}I-insulin to purified receptor with disuccinimidyl suberate as previously described (Pilch & Czech, 1979; Morgan & Roth, 1985). For some procedures, purified receptor was labeled by iodination with Bolton-Hunter reagent (New England Nuclear) to a specific activity of 15 Ci/g.

Antibody production

The monoclonal antibodies discussed in this work were developed in two fusions. For the first fusion, a BALB/c mouse received four intraperitoneal injections of 5-10 μg of receptor emulsified in Freund's complete adjuvant, including a final boost 4 days before fusion. Splenic lymphocytes (5 X 10^7 cells) were fused with Sp2/0 myeloma cells (5 X 10^7 cells) with poly(ethylene glycol) as previously described (Roth et al., 1982) and plated onto 10 24-well culture plates. For the second fusion, an SJL mouse received two intraperitoneal injections of 20-50 μg of receptor emulsified in Freund's complete adjuvant, one similar injection with incomplete Freund's adjuvant, and two intraperitoneal injections of 30 μg of receptor without any adjuvant 5 and 4 days before fusion. Splenic lymphocytes (8 X 10^7 cells) were fused with Sp2/0 myeloma cells (4 X 10^7 cells) and plated onto 30 96-well culture plates. Feeder layers of peritoneal macrophages were used for fused cells and in subsequent clonings by limiting dilution (De St. Groth & Sheidegger, 1980).

Hybridoma supernatants were assayed for anti-receptor antibodies with a plate immunoprecipitation assay described previously (Morgan & Roth, 1985). Briefly,

microtiter wells of flexible poly(vinyl chloride) are coated with 50 µl of rabbit anti-mouse IgG (40 µg/ml). These wells are then incubated with hybridoma supernatants, and the precence of anti-receptor antibodies is detected by adding ^{125}I-insulin-receptor (cross-linked as described above), washing, and counting the wells. Both IgG and IgM anti-receptor antibodies are detected by this method, since the anti-mouse IgG recognizes immunoglobulin light chains as well as the γ heavy chain. To further characterize the antibodies, in some assays the plates were coated with either protein A (50 µg/ml) or anti-IgG-specific antibodies (40 µg/ml). The assay was then performed as usual.

In those studies requiring purified antibodies, the hybridomas were grown as ascites tumors. The IgG antibodies were then purified on a protein A-Sepharose column (Roth et al., 1982), and the IgM antibodies were purified by ammonium sulfate precipitation and gel filtration on a Sepharose 4B column (Roth & Koshland, 1981).

Mapping Antigenic Regions of the Insulin Receptor

A modified plate immunoprecipitation method was used to characterize the competition among antibodies for determinants on the receptor. Wells were coated with rabbit anti-mouse IgG and then hybridoma supernatants as described above. For the purposes of these mapping studies, anti-receptor antibodies bound to the wells in this manner are called "test antibodies". Wells coated with test antibodies were then incubated with cross-linked ^{125}I-insulin-receptor, which had been pre-incubated with excess amounts of a "protecting antibody". Precipitation of the protected receptor by test antibodies was then compared to the precipitation of unprotected receptor, providing the degree of inhibition by the protecting antibody. Preincubation of receptor with excess protecting antibody was accomplished by diluting cross-linked ^{125}I-insulin-receptor 100-fold in hybridoma supernatant from overgrown cells for 90 min at 24°C. Receptor with or without protecting antibody was then added to test-antibody-coated wells for 1 h at 24°C, washed and

counted. Normal mouse serum (1/100) was included in this incubation to prevent binding of protecting antibodies to anti-mouse antibodies on the plate.

Effects of Antibodies on Receptor Kinase Activity

Initial characterization of antibody inhibition of receptor autophosphorylation was accomplished with a modified plate immunoprecipitation assay. Wells were coated with rabbit anti-mouse IgG and the hybridoma supernatants as described above. Meanwhile, 5 µl of purified insulin receptor (250 ng) in buffer A (50 mM Hepes, pH 7.6/150 mM NaCl/ 0.1% Triton X-100) was incubated 1 h at 24°C with 3 ng of ^{125}I-insulin (120 Ci/g). Reactions were then diluted into 50 µl of buffer A containing 1 mg/ml BSA and 2 mM $MnCl_2$ and added to the washed antibody-coated wells for 2 h at 24°C. [γ-^{32}P]ATP (2 µM) was added for 1 h at 24°C, and wells were washed twice with PBS. Proteins bound to the well were counted on a γ counter and subjected to reduced SDS-polyacrylamide gel electrophoresis. With the autoradiograph as a guide, the 95-kDa receptor β subunit was excised and counted in a liquid scintillation counter. Since different amounts of ^{125}I-insulin-bound receptor were precipitated by different antibodies, ^{32}P counts in the β subunit were expressed as a fraction of ^{125}I-insulin bound to the well. Comparison of ^{32}P/^{125}I ratios then provided an estimate of the inhibition of phosphorylation relative to an antibody known to be without effect (1G2).

More detailed characterization of kinase inhibition was carried out with selected purified antibodies. Duplicate 20-µl reaction mixtures contained 250 ng of purified receptor in buffer A, 1 µM insulin, 0.5 mg/ml BSA, and 5 mM $MnCl_2$, plus the desired concentrations of anti-receptor antibody or normal IgG. After a 1 h 24°C incubation, [γ-^{32}P]ATP (5 µM) was added for 1 h at 24°C. Reactions were subjected to reduced SDS-polyacrylamide gel electrophoresis and autoradiography, and the 95-kDa β-subunit band was excised and counted. Exogenous substrate phosphorylation was studied by including 5 µg of histone H2b in the reaction mixtures and excising and counting this band.

Effect of Autophosphorylation on Precipitation of Receptor by Antibodies

Microtiter wells were coated as above with rabbit anti-mouse IgG and hybridoma supernatants. Meanwhile, purified insulin receptor (100 ng) in buffer A was incubated 2 h at 24°C with 1.4 ng of ^{125}I-insulin and 3 mM $MnCl_2$ in the presence or absence of 1 mM ATP. Reactions were diluted 1/20 in 50 µl of buffer A with 0.1% BSA, added to washed antibody-coated wells for 2 h at 24°C, washed, and counted. One series of wells received receptor incubated without ATP, and a duplicate series received receptor incubated with ATP. Differences in the binding of phosphorylated and non-phosphorylated receptor were then expressed as the percent inhibition of receptor precipitation after phosphorylation.

For more detailed studies, wells were incubated overnight at 4°C with rabbit anti-mouse IgG, washed, and incubated 4 h at 24°C with hybridoma 20D9 supernatant. Meanwhile, triplicate 50 µl reaction mixtures containing 100 ng of purified receptor in buffer A were incubated 2 h at 24°C with 1 ng of ^{125}I-insulin, 1 mM $MnCl_2$, 1% BSA, and various concentrations of ATP or AMP-PNP. These mixtures were added to anti-receptor antibody-coated wells for 2 h at 24°C, washed, and counted.

Precipitation of IGF-I Receptor by Antibodies

Binding studies of the purified placental insulin receptor preparation revealed the presence of 10-20% IGF-I receptor (not shown). Thus, this preparation was used in an attempt to identify antibodies to the IGF-I receptor. Two series of microtiter wells were coated with rabbit anti-mouse IgG and hybridoma supernatants. Antibody-coated wells were incubated overnight at 4°C with 100 ng of purified receptor in buffer A with 1% BSA and washed twice. ^{125}I-insulin (45,000 cpm) in buffer A/1% BSA was added to one series of wells and ^{125}I-IGF-I (44,000 cpm) was added to the second series. After 90 min at 24°C, wells were washed and counted. Precipitation of ^{125}I-IGF-I binding activity was expressed as a percentage of ^{125}I-insulin binding activity.

RESULTS

Initial Characterization of Antibodies

Results from the initial screening assays demonstrated the presence of one anti-receptor antibody (7D5) out of 200 cultures from the first fusion and 43 anti-receptor antibodies out of 446 cultures from the second fusion (Table 3.1). Thirty-six of these hybridomas were successfully cultured and characterized. These 36 antibodies, plus a previously described antibody (MC51), comprise the 37 monoclonal antibodies characterized in this work. All 37 of these antibodies precipitated receptor labeled by either cross-linking to ^{125}I-insulin (Table 3.1) or by iodination (data not shown).

Eight of the 37 antibodies were not recognized by IgG-specific anti-mouse antibodies (Table 3.1), and polyacrylamide gel analysis suggested that these eight antibodies were of the IgM class. Further studies with anti-μ chain specific antisera confirmed this identification. Of the remaining 29 antibodies that are of the IgG class, 12 were bound by protein A (Table 3.1).

Mapping Antigenic Regions of the Receptor

To determine how many different antigenic regions of the receptor were recognized by these 37 antibodies, the different antibodies were tested for the ability to compete with each other for binding to the receptor. Receptor was incubated with an excess of protecting antibody and then examined for its ability to be precipitated in the plate assay by the test antibodies (Figure 3.1). Those protecting antibodies that blocked the precipitation of the receptor by the test antibody were considered to bind to the same antigenic "region". Antigenic regions, as defined by Tzartos et al. (1981), may contain numerous antigenic determinants separated by less than the diameter of an antibody arm (35 Å).

For the three antibodies that bind to the extracellular domain of the receptor, three antigenic regions were found (α_1, α_2, and α_3) (Figure 3.2). The two antibodies (MC51 and 5D9) that inhibit insulin binding reacted with closely spaced but not identical regions since MC51 inhibited 5D9 binding by only 30%. The third antibody to the extracellular domain (3D7) did not inhibit insulin binding, and its binding to the receptor was not affected by the other two antibodies (Figure 3.2).

Competition studies with the 34 anti-β antibodies indicated that the cytoplasmic domain of the receptor contained at least three major antigenic regions (β_1, β_2, and β_3) and one minor antigenic region (β_4) (Figure 3.2). Sixteen of the antibodies were found to be directed against β_1, nine against β_2, seven against β_3, and two against β_4 (Figure 3.2). Three antibodies to β_1 (7D5, 13B4, and 30D3) inhibited by more than 70% the binding of the receptor to the remaining antibodies to β_1. These same three antibodies had a smaller effect on the binding of the receptor to the antibodies to β_2 (0-20% inhibition) and no effect on the binding of the receptor to antibodies to β_3. In contrast, 17A3, an antibody to β_2, inhibited by more than 90% the binding of the receptor to antibodies to β_2 but not to the antibodies to β_1 or β_3. Finally, three antibodies to β_3 preferentially inhibited the binding of the receptor to the other antibodies to β_3. The binding of two antibodies (11B11 and 25D4) to the receptor was not inhibited by the antibodies to β_1, β_2, or β_3, and these two antibodies were therefore defined as binding to a fourth region, β_4. The two β_4 antibodies compete with each other for binding to the same region of the receptor and partially inhibit the binding of receptor to antibodies to the β_3 region (data not shown).

Effect of Antibodies on Kinase Activity

In order to identify relationships between antigenic regions and functional domains, we determined the effect of the antibodies on insulin-stimulated receptor autophosphorylation. The effects of the antibodies on autophosphorylation ranged from no inhibition to almost complete inhibition (Figure 3.3A). The nine antibodies to β_2 all inhibited the

autophosphorylation by more than 90%. Results with the antibodies to the other regions were not so clear-cut: some β_1 antibodies were inhibitory (up to 80% inhibition), and others had no effect. The β_3 and β_4 antibodies were all moderately inhibitory (30-85% inhibition).

To determine if differences in the inhibition of autophosphorylation are due to differences in the affinity of antibodies for the receptor, studies of antibody affinity were performed (Figure 3.4). The highly inhibitory β_2 antibodies all half-maximally precipitated the receptor at 2×10^{-9} M (Figure 3.4B). The β_1 antibodies 7D5 and 15B5, which were less effective kinase inhibitors, half-maximally precipitated the receptor at 3×10^{-10} and 10^{-9} M, respectively (Figure 3.4A). Another β_1 antibody, 1G2, which had no effect on the kinase activity, half-maximally precipitated the receptor at 2×10^{-9} M (Figure 3.4A). The β_3 and β_4 antibodies 17H5 and 25D4, which inhibited the kinase by 70 and 25%, respectively, half-maximally precipitated the receptor at 3×10^{-9} and 5×10^{-10} M, respectively (Figure 3.4A). Thus, the different effects on autophosphorylation were not entirely due to the different affinities of the antibodies.

To determine if inhibition of autophosphorylation is accompanied by an inhibition of exogenous substrate phosphorylation, the antibodies were tested for their effects on the ability of the insulin receptor kinase to phosphorylate histone. The antibodies were found to inhibit the phosphorylation of histone as effectively as autophosphorylation. With two β_2 antibodies, half-maximal inhibition of both autophosphorylatyion and histone phosphorylation occurred with the same antibody concentration and to the same maximal extent (Figure 3.5). Similar studies with moderately inhibitory β_1 and β_3 antibodies also demonstrated parallel inhibition of both autophosphorylation and histone phosphorylation (not shown).

Effect of Receptor Autophosphorylation on Binding of Antibodies

Autophosphorylation of the receptor was found to affect the precipitation of the receptor by several antibodies (Figure 3.3B). The nine β_2 antibodies were most affected by autophosphorylation; these antibodies did not precipitate the autophosphorylated receptor. The antibodies to the other regions were less affected by autophosphorylation (precipitation of the receptor by these antibodies was inhibited from 0 to 50%).

More detailed studies with the β_2 antibody 20D9 (Figure 3.6) indicated that half-maximal inhibition of receptor precipitation occurred at 15 μM ATP, a value similar to the K_m for ATP in the autophosphorylation reaction (Petruzzelli et al., 1984). Precipitation of the receptor by 20D9 was not inhibited under conditions where autophosphorylation does not occur: for example, in the presence of ATP but without Mn^{2+} or in the presence of the non-hydrolyzable ATP analogue AMP-PNP and Mn^{2+} (Figure 3.6).

Precipitation of the IGF-I Receptor

Since the IGF-I receptor is structurally (Rechler & Nissley, 1985) and antigenically (Kull et al., 1983; Rosenfeld et al., 1981; Jonas et al., 1982; Roth et al., 1983c; Kasuga et al., 1983b) related to the insulin receptor, the monoclonal antibodies were tested for their ability to bind this receptor. The majority of the antibodies were found to recognize the IGF-I receptor to only a limited extent (Figure 3.3C). However, the nine β_2 antibodies recognized the IGF-I receptor as well as the insulin receptor. No other patterns in the other antigenic groups were apparent.

DISCUSSION

The insulin receptor molecule contains two known functional domains: the insulin binding site on the extracellular surface of the α subunit and the tyrosine-specific protein kinase in the cytoplasmic domain of the β subunit. The aim of the present work was to identify the

antigenic regions of the insulin receptor and the relationship of these regions to the two functional domains of the receptor. To accomplish this, a panel of 37 monoclonal antibodies to the receptor was utilized. These 37 antibodies were shown to precipitate cross-linked ^{125}I-insulin receptor complexes (Table 3.1, Figure 3.6) and purified, iodinated receptor (data not shown). In addition, several of these antibodies have been shown to precipitate insulin receptor from ^{35}S-labeled cell lysates (Roth et al., 1982; Morgan et al., 1986a). Finally, affinity columns composed of several of these antibodies have been shown to purify the insulin receptor from various tissues (Morgan et al., 1986a).

Of these 37 monoclonal antibodies, only 3 were found to bind the extracellular domain of the receptor (Morgan et al., 1986a). The remaining 34 antibodies bind to the intracellular domain of the β subunit. The much greater immunogenicity of this region may have several explanations. First, the intracellular domain of the β subunit may be much more hydrophilic than the rest of the receptor, since hydrophilic portions of proteins are often more immunogenic (Hopp & Woods, 1981). However, the sequence of the insulin receptor indicates that portions of both the α and β subunits are hydrophilic (Ullrich et al., 1985; Ebina et al., 1985b). Recent studies have suggested that highly immunogenic regions of proteins are more mobile than other regions of the molecule (Westhof et al., 1984; Tainer et al. 1984). The cytoplasmic domain of the β subunit may be such a mobile portion of the receptor. Autophosphorylation of the receptor has been shown to lead to activation fo the receptor kinase (Rosen et al., 1983; Yu et al., 1984), and this activation could result, as Hunter has hypothesized for the epidermal growth factor receptor (Hunter, 1984), by the movement of the autophosphorylation site from the active site of the receptor kinase. Finally, antibodies to the extracellular domain of the insulin receptor may be selected against if they cross-react with the mouse insulin receptor. Lymphocytes have insulin receptors (Helderman & Strom, 1978), and insulin has been reported to be necessary for growth of hybridomas in serum-free media (Chang et al., 1980). Thus, a hybridoma that produces a monoclonal antibody that reacts with the mouse insulin receptor might not

survive. That this selection process may be occurring is suggested by the finding that the monoclonal antibodies to the extracellular domain of the receptor are specific for the human receptor whereas antibodies to the intracellular domain cross-react with the rat and mouse receptors (Morgan et al., 1986a).

Due to the relatively low number of antibodies to the extracellular domain of the receptor, extensive characterization of antigenic regions in this domain is not possible. Two major extracellular regions were identified, one of which contains the insulin binding site. The two antibodies to the insulin binding site (MC51 and 5D9) behaved quite differently in the various experiments and thus appeared to recognize different determinants. Antibody 5D9 precipitated over 4-fold greater amounts of cross-linked ^{125}I-insulin-receptor (Table 3.1, Figure 3.4C) and twice as much iodinated receptor as MC51. Thus, 5D9 may bind to a determinant that is more accessible than the MC51 determinant. Also, MC51 only inhibited by 30% the precipitation of receptor by 5D9 (Figure 3.2). Finally, 5D9 inhibited IGF-I binding to IM-9 lymphoid cells 100 times more effectively than MC51 (unpublished studies). The data therefore suggest that these two antibodies bind closely spaced but distinct determinants.

A second region on the extracellular domain of the receptor is recognized by antibody 3D7. This antibody did not inhibit insulin binding and may bind to the same region as the monoclonal antibody described by Kull et al. (1983). In comparison to the other antibodies, this antibody precipitated relatively low amounts of receptor (Figure 3.4C), perhaps because it only recognizes certain glycosylated forms of the receptor. Proteolysis of the β subunit did not affect the binding of the antibody to the receptor (Morgan et al., 1986a), suggesting that the antibody primarily recognizes the α subunit.

Antigenic regions in the cytoplasmic domain of the β subunit have been more extensively characterized in these studies. According to competition experiments, this domain contains three major antigenic regions and one minor region. This would agree with the four to six antigenic regions expected on the surface of a globular protein the size

of the β-subunit cytoplasmic domain (40 kDa). Each antigenic region may contain several antigenic determinants. For example, antibodies to the β_1 region form a particularly heterogeneous population. β_1 antibodies vary considerably in their capacity and affinity for the receptor (Figure 3.4A), their effect on the kinase activity of the receptor (Figure 3.3A), their affinity for autophosphorylated receptor (Figure 3.3B), and their ability to compete with other antibodies for receptor binding (Figure 3.2). These differences suggest that the β_1 region contains more than one determinant.

Antibodies to the β_3 region are slightly heterogeneous in their behavior in antigenic mapping studies (Figure 3.2) and affinity and capacity for receptor (Table 3.1), but they appear similar in their effect on the kinase (all moderate inhibitors, Figure 3.3A) and affinity for the autophosphorylated receptor (all slightly inhibited by autophosphorylation, Figure 3.3B). The β_3 region thus appears to be composed of a more limited number of determinants that are more closely associated with the kinase activity of the receptor than the β_1 antibodies.

Antibodies to the β_2 region gave similar results in all experiments: all nine of these antibodies inhibited the receptor kinase by more than 90%, did not recognize phosphorylated receptor, and recognized the IGF-I receptor very well (Figure 3.3). Evidence for some heterogeneity of the β_2 class was found in the antigenic mapping studies (Figure 3.2), where the binding of four of the β_2 antibodies was more easily inhibited by β_1 and β_3 antibodies. One of these four β_2 antibodies (24B7) was compared to one of the other β_2 antibodies (17A3) in its effect on kinase activity (Figure 3.5), and slight differences in affinity and maximal inhibitory effect were observed. However, despite these minor variations within the β_2 class, it is clear that the antibodies to the β_2 region are a less heterogeneous group than the antibodies to the β_1 and β_3 regions. These results would suggest that the β_2 region is composed of a limited number of antigenic determinants.

The observation that β_1 and β_3 antibodies partially compete with β_2 antibodies and not with each other (Figure 3.2) would suggest that the β_2 region lies between the β_1 and β_3 regions. It is therefore surprising that a β_2 antibody did not affect the binding of receptor to β_1 or β_3 antibodies. One explanation for this apparent discrepancy is that β_1 and β_3 antibodies inhibit β_2 antibody binding by mechanisms other than steric hindrance; instead, they may induce conformational changes in the receptor that reduce the affinity of β_2 antibodies for the receptor. Another explanation for why the β_2 antibodies do not affect antibody binding to the β_1 and β_3 regions is that they bind to a flexible structure that moves away from adjacent regions after binding to the β_2 antibody. As mentioned above, activation of the receptor kinase by autophosphorylation may involve such a movement on the receptor molecule. Thus, the β_2 antibodies may recognize a flexible autophosphorylation domain on the insulin receptor. This possibility is supported by the observatiion that the β_2 antibodies completely inhibited receptor autophosphorylation and did not recognize receptor after incubation with ATP and Mn^{2+} (Figure 3.3B). This effect of ATP and Mn^{2+} on antibody recognition appeared to result from the autophosphorylation reaction because (1) the half-maximal concentration of ATP in the effect was the same as the K_m for ATP in the autophosphorylation reaction (Petruzzelli et al., 1984) and (2) the effect did not occur under conditions where autophosphorylation does not occur, such as in the presence of ATP without Mn^{2+} or in the presence of a nonhydrolyzable ATP analogue, AMP-PNP, and Mn^{2+}. Interestingly, the carboxy-terminal domain of the insulin receptor β subunit is highly hydrophilic (Ullrich et al., 1985) and, therefore, may be an important antigenic region. In addition, it has been suggested by Hunter that this region of the insulin receptor contains the sites of autophosphorylation (Hunter, 1985); thus, it is conceivable that the β_2 antigenic region includes the carboxy-terminal domain of the β subunit.

Identification of the amino acid sequences that comprise the different antigenic regions may be complicated by the finding that all the present monoclonal antibodies bind to conformation-dependent determinants. Thus, none of these antibodies bind to receptor after

it has been denatured in sodium dodecyl sulfate. An alternative approach for identifying the important amino acid sequences in each antigenic region may be to study the binding of the antibodies to homologous proteins. For example, the finding that the antibodies to β_2 all react equally with the IGF-I and insulin receptors suggests that this portion of the insulin receptor should be highly homologous to the corresponding portion of the IGF-I receptor. Further studies with other homologous proteins, such as the tyrosine kinase product of the avian sarcoma virus UR2 (ros)(Ebina et al., 1985b; Feldman et al., 1982; Neckameyer & Wang, 1985), may identify other cross-reactions that can be used to further determine the protein sequences that comprise the various antigenic regions.

Table 3.1. Properties of monoclonal antibodies to the insulin receptor.

Antibody	^{125}I-insulin-receptor precipitation by antibodies bound to microtiter wells coated with: anti-mouse Ig (cpm)	Protein A (cpm)	anti-mouse IgG (cpm)	heavy chain class
MC51	260	180	310	γ
5D9	2220	370	2250	γ
3D7	2690	2120	2110	γ
1C1	1960	60	80	μ
1G2	1720	1090	1450	γ
7D5	3080	60	100	μ
8H2	2850	770	2790	γ
13B4	2300	10	50	μ
15B5	2760	1230	2680	γ
16E8	2850	2460	2550	γ
17G6	1380	20	50	μ
19E3	1040	220	770	γ
20B4	1390	820	1390	γ
20G2	3290	1630	3070	γ
21C11	2290	860	2100	γ
28F2	3110	730	3100	γ
29B4	3450	2500	3510	γ
30D1	570	140	500	γ
30D3	2290	820	2300	γ
4E10	1580	40	40	μ
16F5	920	10	50	μ
17A3	2120	1940	1980	γ
24D5	290	80	360	γ
28B7	2210	2360	1970	γ
17E5	2400	1470	2300	γ
20D9	2060	540	2200	γ

(continued next page)

Table 3.1. Properties of monoclonal antibodies to the insulin receptor (continued).

Antibody	^{125}I-insulin-receptor precipitation by antibodies bound to microtiter wells coated with: anti-mouse Ig (cpm)	Protein A (cpm)	anti-mouse IgG (cpm)	heavy chain class
21D3	2820	740	2580	γ
24B7	2560	940	2480	γ
2G7	3750	2160	3080	γ
3F10	2970	1400	3050	γ
17H5	1640	460	1390	γ
19H9	1470	410	1240	γ
25D8	1200	180	30	μ
27B2	1700	1310	1560	γ
29E3	3650	2590	3890	γ
11B11	280	1890	40	γ
25D4	1990	50	140	μ

Microtiter wells were coated with rabbit anti-mouse IgG, protein A, or goat anti-mouse IgG (Fc fragment specific), as described under Experimental Procedures. After being washed, wells were incubated 4 h at 24°C with hybridoma supernatants, followed by a wash and a similar incubation with cross-linked ^{125}I-insulin-receptor (8000 cpm). Wells were washed and counted. Heavy chain class was supported by sodium dodecyl sulfate-polyacrylamide gel analysis and further confirmed in studies using plates coated by IgM-specific antibodies. MC51 is the previously described monclonal antibody to the insulin binding site. Results shown are representative of several experiments; interassay variability was less than 10%.

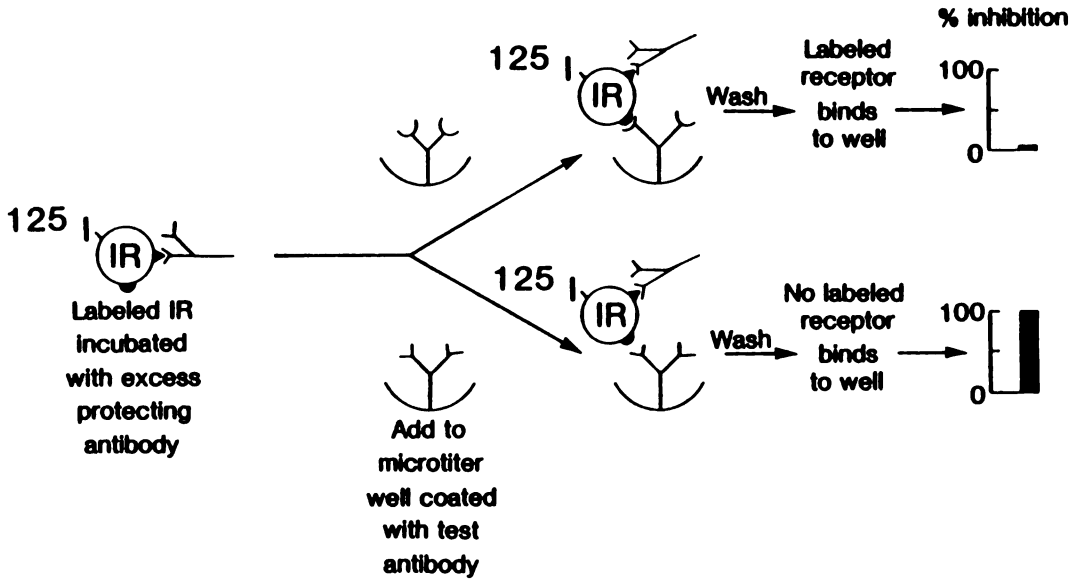


Figure 3.1. Method for mapping antigenic regions of the insulin receptor.

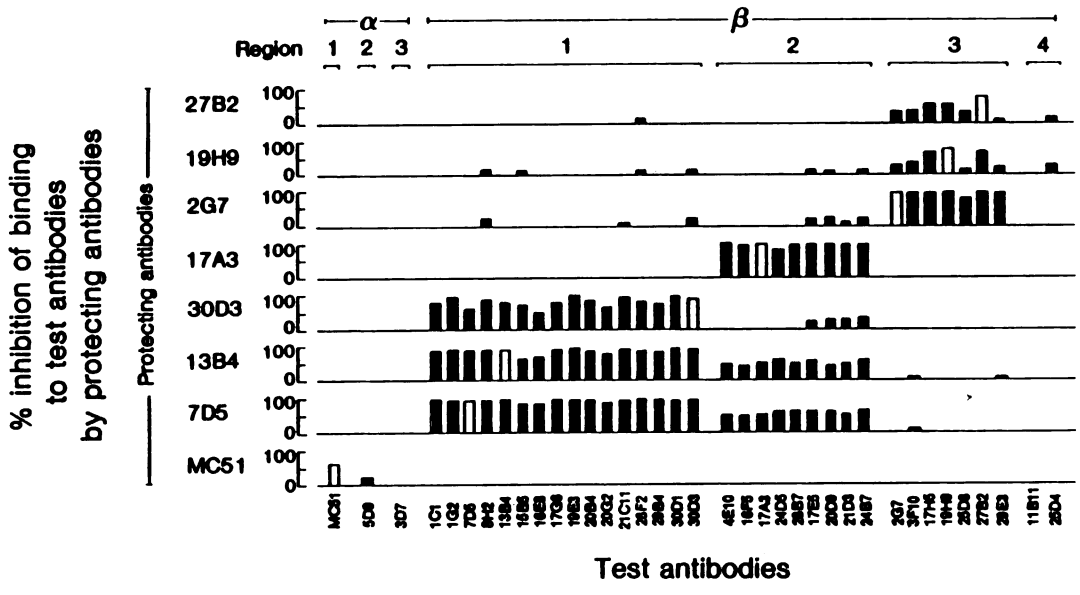


Figure 3.2. Antigenic regions of the insulin receptor.

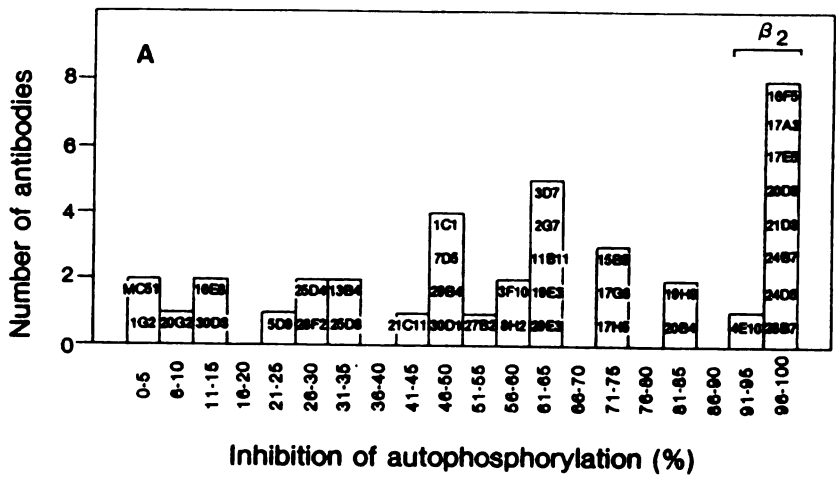
As outlined in Figure 3.1, labeled receptor was preincubated with the indicated protecting antibodies and then examined for binding to microtiter wells coated with the indicated test antibodies. The bars indicate the percent inhibition of receptor precipitation caused by the protecting antibody; this inhibition varied by less than 10% between experiments.

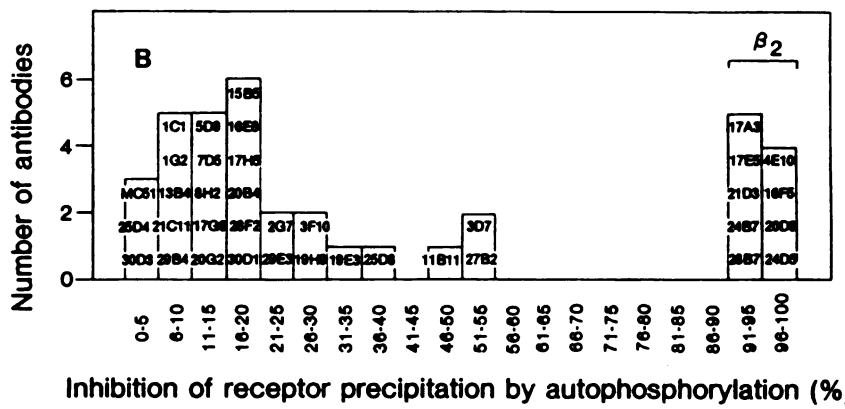
Figure 3.3 (next page). Mapping antigenic regions of the insulin receptor by functional assays.

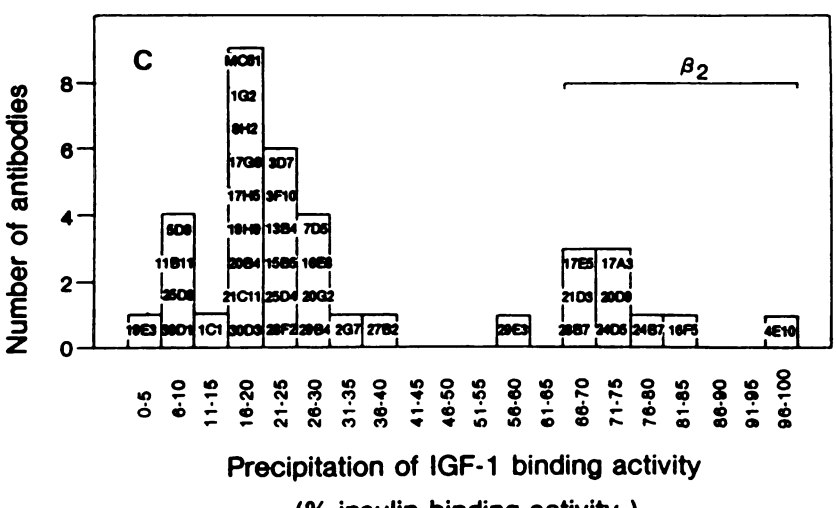
(A) Effect of antibodies on receptor autophosphorylation. The amount of ^{32}P incorporated into the β subunit of receptor precipitated by antibody-coated microtiter wells was determined. Values were normalized to the amount of receptor present as determined by ^{125}I-insulin binding. Percent inhibition was calculated by comparison with antibody 1G2, which has no effect on the receptor kinase (determined in separate experiments with purified 1G2).

(B) Effect of receptor autophosphorylation on precipitation by antibodies. Precipitation of autophosphorylated ^{125}I-insulin-bound receptor by antibody-coated microtiter wells was compared to precipitation of non-phosphorylated receptor as described under Experimental Procedures. Data are expressed as inhibition of receptor precipitation due to autophosphorylation.

(C) Precipitation of ^{125}I-IGF-I binding activity by antibodies. Precipitation of ^{125}I-IGF-I binding activity by antibody-coated microtiter wells was expressed as a percentage of the precipitation of ^{125}I-insulin binding activity, as described under Experimental Procedures. Results in panels A-C are representative of several experiments. Results of inhibition of autophosphorylation (panel B) varied by 20% between experiments, whereas the other results varied by less than 10%.


A
β2
Number of antibodies
0
2
4
6
8
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
Inhibition of autophosphorylation (%)


B
β2
Number of antibodies
0
2
4
6
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
Inhibition of receptor precipitation by autophosphorylation (%)


C
β2
Number of antibodies
0
2
4
6
8
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
Precipitation of IGF-1 binding activity
(% insulin binding activity)

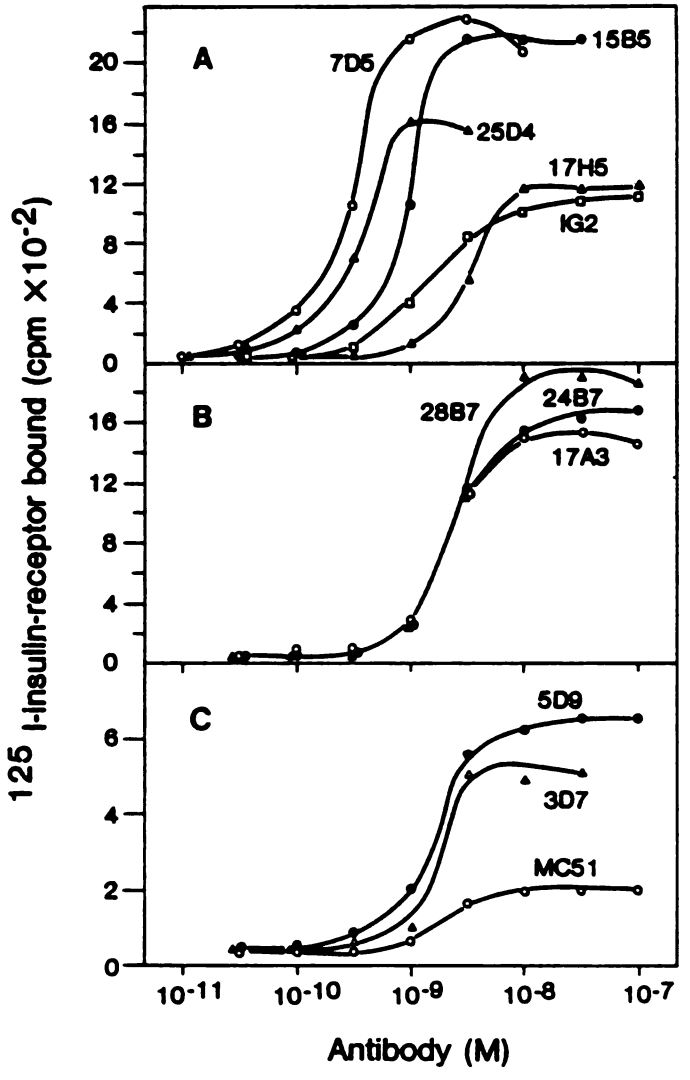


Figure 3.4. Precipitation of insulin receptor by different concentrations of several antibodies.

Precipitation of cross-linked ^{125}I-insulin-receptor by microtiter wells coated with the indicated amount of antibodies was performed with the plate precipitation assay described under Experimental Procedures. ^{125}I-insulin-receptor (8000 cpm) was added to the antibody-coated wells and incubated 4 h at 24°C, followed by washing and counting. (A) β_1 antibodies 7D5 (o), 15B5 (•), and 1G2 (□); β_3 antibody 17H5 (Δ); β_4 antibody 25D4 (▲). (B) β_2 antibodies 17A3 (o), 24B7 (•), and 28B7 (Δ). (C) α antibodies MC51 (o), 5D9 (•), and 3D7 (Δ). Similar results (±5%) were obtained in two separate experiments.

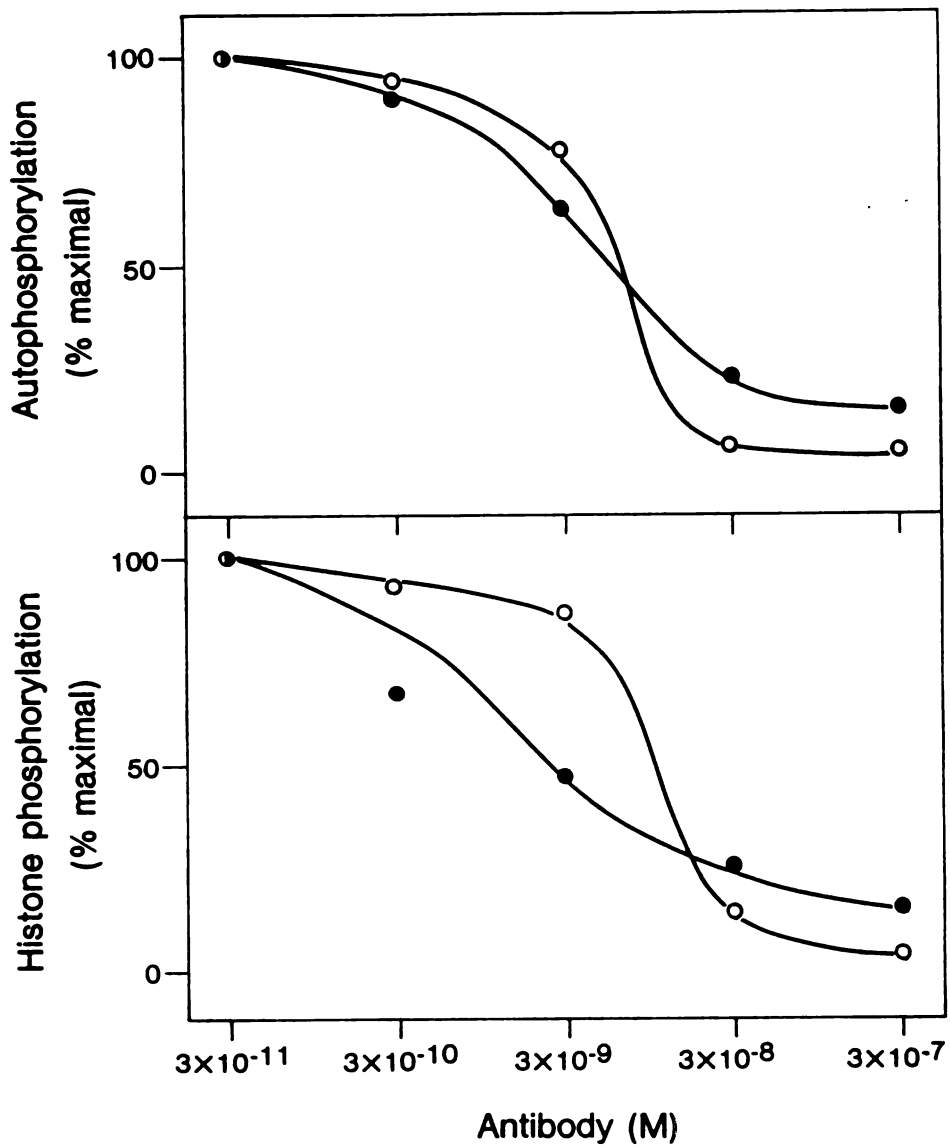


Figure 3.5. Effect of two β_2 antibodies on receptor kinase activity.

Purified insulin receptor autophosphorylation (top) and histone H2b phosphorylation (bottom) were determined in the presence of the indicated concentration of antibodies 17A3 (o) and 24B7 (•), as described under Experimental Procedures. Similar results (±5%) were obtained in three separate experiments.

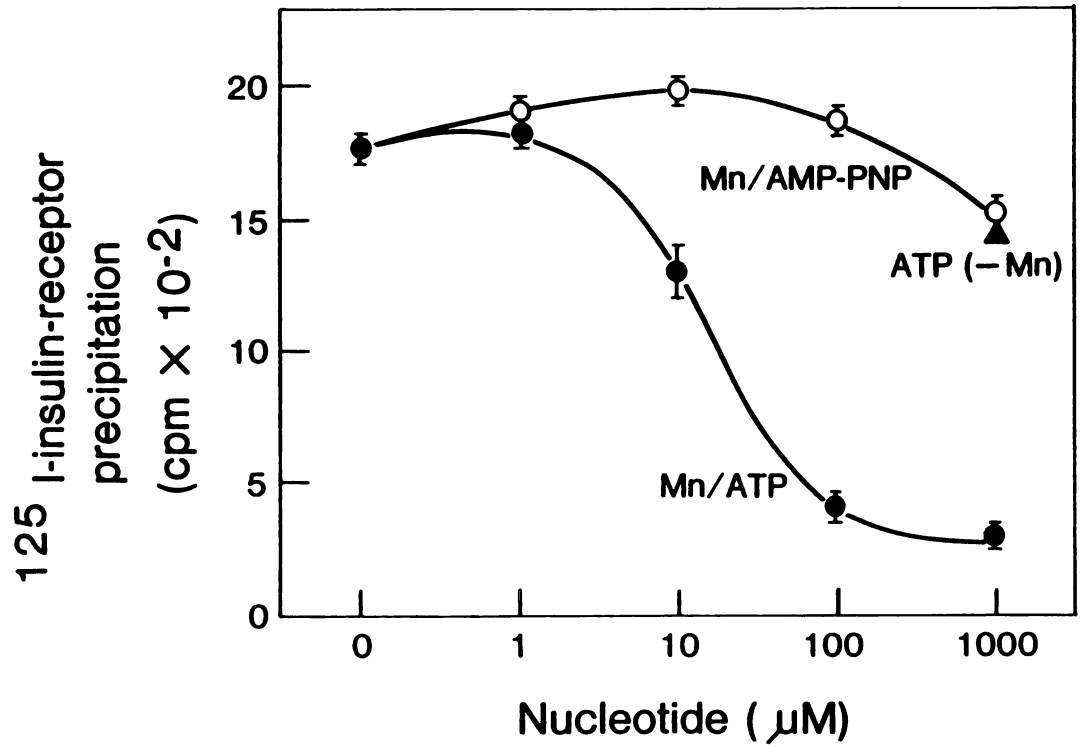


Figure 3.6. Effect of receptor phosphorylation on precipitation by β_2 antibody 20D9.

Precipitation by 20D9-coated wells of receptor preincubated with indicated concentrations of Mn/ATP (●), Mn/AMP-PNP (○), or ATP alone (△) was performed as described under Experimental Procedures. Values are means of triplicates ± S.E.M.

CHAPTER 4

Purification and Characterization of the Receptor for Insulin-like Growth Factor I

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Biochemistry, in press.

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One of the conclusions of the previous chapter was that an important region in insulin receptor kinase activity, the β_2 region, is highly conserved in the closely related receptor for IGF-I. In the present chapter, this property of the β_2 antibodies was used to develop an immunoaffinity technique for purifying the IGF-I receptor to homogeneity. Studies of this purified receptor and its interaction with the β_2 antibodies demonstrate the close relationship between the receptors for insulin and IGF-I.

SUMMARY

The receptor for insulin-like growth factor I (IGF-I) was purified from the rat liver cell line BRL-3A by a combination monoclonal anti-receptor antibody affinity column and a wheat germ agglutinin column. Analyses of these receptor preparations on reduced sodium dodecyl sulfate polyacrylamide gels yielded protein bands of M_r=136 K (α subunit) and M_r=85 and 94 K (β subunit). These receptor preparations bound five times more IGF-I than insulin and the binding of both ligands was more potently inhibited by unlabeled IGF-I than by insulin. These results indicate that these receptor preparations contained predominantly the IGF-I receptor. This highly purified receptor preparation was found to possess an intrinsic kinase activity; autophosphorylation of the receptor β subunit was stimulated by low concentrations of IGF-I (half-maximal stimulation at 0.4 nM IGF-I). Twenty-fold higher concentrations of insulin were required to give comparable levels of stimulation. A monoclonal antibody that inhibits the receptor kinase was found to inhibit the IGF-I receptor kinase with the same potency with which it inhibits the insulin receptor. In contrast, monoclonal antibodies to other parts of the insulin receptor only poorly recognized the IGF-I receptor. A comparison of V8 protease digests of the insulin and IGF-I receptors again revealed some similaritites and also some differences in the structures of these two receptors. Thus, the IGF-I receptor is structurally, antigenically, and functionally similar but not identical to the insulin receptor.

INTRODUCTION

Insulin-like growth factor I (IGF-I) is a polypeptide hormone whose amino acid sequence is about 50% homologous to that of proinsulin (Rinderknecht & Humbel, 1978). IGF-I, at high concentrations, can also bind to the insulin receptor and elicit biological responses through this receptor with about 1% of the potency of insulin (Froesch et al., 1985). In addition, various cells have a distinct receptor for IGF-I which binds IGF-I with high affinity (Rechler & Nissley, 1985). *In vivo*, IGF-I appears to be a primary regulator of growth, whereas insulin primarily functions as a regulator of more acute metabolic responses. However, with cells in culture, examples have been found of insulin regulating cellular growth through its own receptor and IGF-I regulating acute metabolic responses through its distinct receptor (Froesch et al., 1985; Rechler & Nissley, 1985). The different effects of these hormones on various cell types may in part be determined by the levels of the two receptor types on a particular cell.

In addition to sharing a similar spectrum of biological responses and a similar amino acid sequence, IGF-I and insulin have receptors with a similar general structure (for review, see Rechler & Nissley, 1985). Both receptor have α subunits ($M_r \approx 130$ K) which are readily labeled by crosslinking to their respective radioligands. By immunoprecipitation of either biosynthetically or surface labeled cells, both receptors have also been shown to contain a β subunit of $M_r \approx 95$ K. In both receptor types, these two subunits are linked via disulfide bonds to form tetrameric ($\alpha_2\beta_2$) complexes of $M_r \approx 350$ K. Both receptors are synthesized as a single precursor polypeptide of $M_r = 190$ K which is processed to yield the α and β subunits. By analogy to the insulin receptor, it has also been argued that the IGF-I receptor has an intrinsic tyrosine-specific kinase activity since: (i) membrane preparations of a rat liver cell line which are high in IGF-I receptors but low in insulin receptors exhibit an IGF-I stimulatable phosphorylation of a $M_r = 98{,}000$ protein (presumably the β subunit

of the IGF-I receptor) (Sasaki et al., 1985): (ii) these same preparations exhibit an IGF-I stimulated phosphorylation of a tyrosine-containing synthetic substrate (Zick et al., 1984b): (iii) in placenta extracts IGF-I stimulates phosphorylation of the β subunit of the IGF-I receptor with half-maximal stimulation occurring at concentrations of IGF-I which half-maximally inhibit binding of ^{125}I-IGF-I to its receptor (Rubin et al., 1983); and (iv) immunoprecipitates of IGF-I receptor retain kinase activity (Jacobs et al., 1983b).

These results support but do not prove the hypothesis that the IGF-I receptor has an intrinsic kinase activity . First, these studies utilized receptor preparations that were fairly impure (less than 1% of the protein present was the receptor)(Rubin et al., 1983; Sasaki et al., 1985; Zick et al., 1984b). Second, in these various experiments insulin was observed to be more potent at stimulating phosphorylation of both the IGF-I receptor and exogenous substrates than would be expected on the basis of its ability to bind to the IGF-I receptor (Rubin et al., 1983; Sasaki et al., 1985; Zick et al., 1984b). In addition, there was no detectable stimulation of kinase activity by IGF-I in the experiments with isolated IGF-I receptor in immunoprecipitates (Jacobs et al., 1983b). Finally, recent studies of the characteristics of the IGF-I receptor kinase indicated that it was very similar to the insulin receptor kinase in terms of substrate specificity, activation by autophosphorylation, and nucleotide and cation preference (Sasaki et al., 1985). These studies raise the possibility that the presence of insulin receptor in these partially purified preparations was responsible for the observed kinase activity.

Additional evidence to support the hypothesis that the IGF-I receptor is a kinase required its purification to homogeneity. Recently, we developed a panel of monoclonal antibodies to the cytoplasmic domain of the insulin receptor (Morgan et al., 1986a; Morgan & Roth, 1986). Immunoaffinity purification with several of these antibodies has provided highly purified insulin receptor preparations from a variety of species and tissue types (Morgan et al., 1986a; Roth et al., 1986). Since several of these antibodies were found to have high affinity for the IGF-I receptor, it was possible to utilize these antibodies to purify

the IGF-I receptor. Since these same antibodies also bind the insulin receptor, a rat liver cell line (BRL-3A) was used as the source of receptors since it has few insulin receptors but abundant IGF-I receptors (Sasaki et al., 1985). The IGF-I receptor from these cells was purified to homogeneity and found to have an IGF-I stimulatable kinase activity and a structure which is similar to but distinct from that of the insulin receptor.

EXPERIMENTAL PROCEDURES

Materials

Pork insulin was purchased from Elanco and IGF-I (prepared by recombinant DNA technology) was a gift of J. Merryweather, Chiron Corp. Insulin and IGF-I were iodinated by the following procedure to specific activities of 50-100 Ci/g. One to five μg hormone in 20 μl 0.5 M $NaPO_4$ pH 7.4 was combined with 10 μl (1 mCi) [^{125}I]NaI and 20 μl chloramine-T (0.06 mg/ml) in the same buffer. After 30 min at 24°C, labeled hormone was separated from free NaI by passage over a 9 ml Sephadex G-25 column. [γ-^{32}P]ATP (3000 Ci/mmol) is from Amersham; reagents for polyacrylamide gel electrophoresis are from Bio-Rad. Monoclonal antibodies to the insulin receptor have been characterized previously (Morgan et al., 1986a; Morgan & Roth, 1986). All other materials were obtained as in Morgan & Roth (1985, 1986). Rat liver insulin receptor was purified as described (Morgan et al., 1986a; Roth et al., 1986).

Receptor Purification

Confluent roller bottles of BRL-3A cells were solubilized in a minimal volume of ice-cold 50 mM Hepes pH 7.6, 1% Triton X-100, 1 mg/ml bacitracin, 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were clarified by centrifugation (30,000 x g, 3 h, 4°C) and loaded onto a 10 ml Affigel column composed of ten monoclonal anti-receptor antibodies as described (Morgan et al., 1986a). The column was washed with buffer A (50 mM Hepes

pH 7.6, 150 mM NaCl, 0.1% Triton X-100) containing 1 mg/ml bacitracin and 1 mM PMSF, and then with buffer A containing 1 M NaCl. Receptor was eluted with 1.5 M $MgCl_2$, 120 mM sodium tetraborate, 0.1% Triton X-100, pH 6.5, diluted ten-fold in buffer A and loaded onto a 2 ml wheat germ agglutinin (WGA) Affigel column. After washing the column with buffer A, the receptor was eluted with 0.3 M N-acetyl-D-glucosamine in buffer A. Preliminary studies of these preparations indicated the presence of a small amount of insulin receptor, which was removed by passing the preparation over a 2 ml Affigel column composed of monoclonal anti-receptor antibody 15B5, which has a high affinity for the insulin receptor and a low affinity for the IGF-I receptor. Purified IGF-I receptor and rat liver insulin receptor were iodinated with Bolton Hunter reagent (NEN) to a specific activity of approximately 10 Ci/g.

Ligand Binding Studies

IGF-I and insulin binding were measured on purified receptor immobilized on microtiter wells coated with anti-receptor antibodies. Wells of a 96-well poly(vinyl chloride) plate were coated with 50 µl rabbit anti-mouse IgG (10 µg/ml in 20 mM $NaHCO_3$, pH 9.6) for 4 hours at 24°C. Wells were washed twice with wash buffer (20 mM $NaPO_4$ pH 7.4, 150 mM NaCl, 0.05% Tween-20, 0.1% bovine serum albumin, BSA) and incubated 4 hours at 24°C with 50 µl monoclonal antibody 17A3 (3×10^{-8} M) in buffer A containing 1% BSA. Wells were washed twice and incubated 60 min at 24°C with various concentrations of unlabeled ligand, followed by the addition of ^{125}I-IGF-I or ^{125}I-insulin (50,000 cpm) for 90 min at 24°C. Wells were washed twice, cut off, and counted.

Receptor Autophosphorylation

Reaction mixtures of 20 µl buffer A containing 0.1 mg/ml BSA and 5 to 10 ng purified receptor were incubated 60 min at 24°C with 2 mM $MnCl_2$ and desired concentrations of IGF-I or insulin, followed by a 60 min incubation at 24°C with 10 mM [γ-^{32}P]ATP (20-

40 Ci/mmol). Reactions were then analyzed by polyacrylamide gel electrophoresis and autoradiography. The 85-94 kDa β-subunit bands were excised and counted. The effect of antibody 17A3 on kinase activity was measured by including the desired concentration of protein A purified 17A3 in the initial 60 min incubation.

Protease Mapping

^{125}I-labeled receptor preparations (600,000 cpm) were subjected to polyacrylamide gel electrophoresis after denaturation and reduction by boiling in 1% SDS and 5% 2-mercaptoethanol. The separated α and β subunits were excised from the unfixed gel, using pre-stained molecular weight markers (BRL) as a guide. Proteins were eluted from crushed gel slices by soaking 16 hours at 4°C in 200 µl buffer A and then centrifuging to pellet the gel. Aliquots (20 µl) of these eluted subunits were then combined with 10 µl buffer A containing the desired concentration of *Staphylococcus aureus* V8 protease (Sigma). After 30 min at 37°C, reactions were analyzed by electrophoresis on 15% polyacrylamide gels.

RESULTS

IGF-I Receptor Structure

Soluble lysates of the rat liver cell line BRL-3A (which are high in IGF-I receptor) were purified by sequential affinity chromatography on a column composed of monoclonal anti-receptor antibodies and a wheat germ agglutinin column. Small amounts of insulin receptor in these IGF-I receptor preparations were removed by passage of the material over an affinity column containing an antibody which preferentially binds the insulin receptor. The purified IGF-I receptor was compared to the rat liver insulin receptor by ^{125}I-labeling and analysis by SDS gel electrophoresis and autoradiography (Figure 4.1). The two rat receptor types had a similar pattern of two major subunits: one at M_r=136 K and a doublet at roughly 85 and 94 K. These molecular weights correspond to previously known molecular

weights of the receptor α and β subunits, respectively. Slight differences in molecular weights were observed between the insulin and IGF-I receptor β subunits. Since insulin receptors from different tissues also exhibit slightly different molecular weights on SDS gels, it is not possible to conclude solely from the M_r which receptor is the IGF-I receptor and which is the insulin receptor.

IGF-I Binding Activity

To distinguish between the receptors for insulin and IGF-I, binding studies were performed with ^{125}I-labeled IGF-I and insulin. The BRL-3A receptor preparation was found to bind five times more labeled IGF-I than insulin (Figure 4.2A). Since IGF-I receptors also have a weak affinity for insulin, competition studies were performed to determine whether the observed binding of ^{125}I-insulin was due to the presence of a small amount of insulin receptor or ^{125}I-insulin binding to the IGF-I receptor. The binding of ^{125}I-IGF-I to the BRL-3A preparation was displaced with high affinity by IGF-I (half-maximal inhibition at 0.6 nM IGF-I) and displaced poorly by insulin (I_{50}=30 nM insulin); thus, a high affinity IGF-I receptor binding site is present in these preparations. Scatchard analysis of IGF-I binding data (not shown) indicates that the receptor preparations bound approximately 0.5 to 1.5 moles IGF-I per mole receptor. The small amount of ^{125}I-insulin binding to this receptor preparation was displaced by low concentrations of IGF-I (I_{50}=0.5 nM) and high concentrations of insulin (I_{50}=7 nM), indicating that the observed binding of labeled insulin was also to the IGF-I receptor.

In contrast to the results with the receptor preparations from BRL-3A cells, the rat liver receptor preparations contained mainly the insulin receptor. The rat liver preparations bound 5 times more insulin than IGF-I and the ^{125}I-insulin binding was displaced by low concentrations of insulin (I_{50}=9 nM insulin) and not displaced by IGF-I at concentrations of 10 nM (Figure 4.2B). Rat liver insulin receptor preparations also bound a small amount of ^{125}I-IGF-I. This binding appeared to be due to the presence of a small amount of IGF-I

receptor in these preparations, since IGF-I was more potent than insulin at inhibiting its binding (Figure 4.2B).

Receptor Autophosphorylation

Studies of receptor autophosphorylation were then performed to determine if this highly purified IGF-I receptor preparation possessed intrinsic kinase activity. Incubation of this preparation with Mn^{2+}, [γ-^{32}P]ATP, and increasing concentrations of IGF-I resulted in a dose-dependent increase in the labeling of the receptor β subunit (Figure 4.3). The two bands of the β-subunit doublet were stimulated equally. The response to IGF-I was quite sensitive: half-maximal stimulation occurred at 0.4 nM IGF-I, a value close to the potency of IGF-I in the displacement of ^{125}I-IGF-I binding to this receptor. Insulin was over 17-fold less potent in stimulating autophosphorylation; half-maximal stimulation occurred at about 7 nM, a value in close agreement with the ability of insulin to inhibit ^{125}I-insulin binding to this receptor. The maximum incorporation of phosphate into the receptor was approximately 2 to 4 moles phosphate per mole receptor.

Receptor Antigenic Structure

Previous studies with a panel of monoclonal antibodies to the insulin receptor kinase domain have shown that only one of the four major antigenic regions in this domain is conserved in the IGF-I receptor (Morgan & Roth, 1986). Antibodies to this region, the β_2 antibodies, were found to bind the IGF-I receptor to the same extent as they bound the insulin receptor (Morgan & Roth, 1986). Since the antibodies to the β_2 region are potent inhibitors of receptor autophosphorylation, it was possible in the present work to more carefully compare their affinity for the insulin and IGF-I receptors by analyzing their dose-dependent inhibition of receptor autophosphorylation. The β_2 antibody 17A3 inhibited the phosphorylation of the purified IGF-I receptor with the same affinity as it inhibited the rat

liver insulin receptor: both receptor types were inhibited half-maximally at an antibody concentration of 4 nM (Figure 4.4).

Protease Mapping

Structural relationships between the receptors for IGF-I and insulin were further studied by analyzing the pattern of fragments generated by limited proteolysis of the two receptor types. The ^{125}I-labeled α and β subunits of each receptor type were separated on SDS polyacrylamide gels, eluted from the gel, treated with various concentrations of *Staphylococcus aureus* V8 protease, and electrophoresed on gels. The resulting patterns of proteolytic fragments obtained from the two receptor types were remarkably similar, although some small differences in fragment size and number were observed (Figure 4.5). For example, the 26 kDa α-subunit fragment was more heterogeneous in the IGF-I receptor (Figure 4.5A, lanes f, l) and the ≈60 kDa β-subunit fragment of the IGF-I receptor had a slightly lower mobility than the corresponding fragment of the insulin receptor (Figure 4.5B, lanes d, j).

DISCUSSION

In the present work, we have purified the IGF-I receptor from the BRL-3A cell line by utilizing a monoclonal antibody affinity column. The purified receptor preparation bound approximately 5 times more labeled IGF-I than insulin. Moreover, the binding of both of these ligands was 20 to 50 times more potently inhibited by IGF-I than insulin. These results indicate that there was no detectable insulin receptor in these preparations. These purified preparations of IGF-I receptor were found to have an IGF-I stimulatable kinase activity. The concentration of IGF-I which half-maximally stimulated the autophosphorylation reaction (0.4 nM) was close to the concentration of IGF-I which half-maximally inhibited labeled IGF-I binding (0.6 nM). Although insulin also stimulated the

autophosphorylation reaction, the concentration of insulin required for half-maximal stimulation was about 20 times greater than the required concentration of IGF-I. Moreover, the concentration of insulin required to half-maximally stimulate autophosphorylation (7 nM) was the same as the concentration of insulin required to half-maximally inhibit the binding of insulin to the IGF-I receptor (7 nM). These results indicate that insulin is acting through the IGF-I receptor. These results are in contrast to the prior results using less purified receptor preparations where it was found that insulin was only two or three times less potent than IGF-I at stimulating phosphorylation (Zick et al., 1984b; Sasaki et al., 1985). It may be that these earlier studies are complicated by the presence of contaminating insulin receptor.

Polyacrylamide gel analyses of the highly purified IGF-I receptor from the rat hepatoma cells indicates that the IGF-I receptor is composed of two major subunits: α (M_r=136 K) and a doublet of β (M_r=85 and 94 K). This structure agrees with the previously derived structure of the IGF-I receptor from human IM-9 lymphocytes, human placenta, and various cell lines (Rechler & Nissley, 1985). The β-subunit band of the IGF-I receptor in IM-9 lymphocytes was also found to be a doublet (Jacobs et al., 1983b). One of these two bands may have represented a small amount of contaminating insulin receptor. However, this appears unlikely in the present studies since the two bands of the β-subunit doublet are present in equal amounts, and since their autophosphorylation is equally stimulated by IGF-I. Instead, these two bands could result from a partial proteolysis of the β subunit or differential glycosylation. It is even possible that these two bands may represent different IGF-I receptors since evidence has been presented which suggests that several forms of this receptor exist (Jonas & Harrison, 1985).

In addition to sharing a structural and functional similarity with the insulin receptor, the IGF-I receptor also shares antigenic cross-reactivity with antibodies to the insulin receptor. Prior studies with polyclonal antibodies from various patients had indicated that some of these antisera reacted with both the IGF-I and insulin receptors, whereas others reacted

with only the insulin receptor (Kasuga et al., 1983b; Jonas et al., 1982). In these studies it was not possible to determine whether the same antibody molecules were reacting with both receptors. More recently, several monoclonal antibodies to extracellular determinants of the insulin receptor were also shown to react with the IGF-I receptor (Roth et al., 1983c; Kull et al., 1983). However, these antibodies had a 100- to 200-fold higher affinity for the insulin receptor than the IGF-I receptor. In prior studies we examined the binding of the IGF-I receptor by monoclonal antibodies to four distinct antigenic regions of the cytoplasmic domain of the β subunit of the insulin receptor (Morgan & Roth, 1986). Antibodies to one particular region, the β_2 region, were found to precipitate the two receptors equally. In the present studies, antibodies to this region were also found to inhibit with identical potencies the kinase activities of the two receptors. Since the β_2 region is intimately involved in the kinase activity of the receptor, these studies suggest that this region of the the two receptors is most highly related. Recently, it has become possible to identify the residues involved in the β_2 region. Site-directed mutagenesis was used to construct mutant insulin receptor cDNA's for expression in Chinese hamster ovary cells (Ellis et al., 1986). Insulin receptors in which tyrosines 1162 and 1163 were replaced with phenylalanines were recognized very poorly by antibodies to the β_2 region, although antibodies to other antigenic regions recognized these mutated receptors normally. The kinase activity of this mutant receptor, both *in vivo* and *in vitro*, was also severely reduced. Clearly, the β_2 region is an important region in insulin receptor kinase function; the present results demonstrate that the antigenic region recognized by the β_2 antibodies is highly conserved in the IGF-I receptor. Similarly, anti-peptide antibodies directed against another potentially important region of the insulin receptor kinase (residues 952-967) have recently been shown to cross-react with the IGF-I receptor (Herrera et al., 1986). That the kinase activities of the two receptors are highly related is also suggested by studies indicating that the two receptor kinases share substrate specificities, as well as nucleotide and cation preference (Sasaki et al., 1985). Interestingly, the same β_2 antigenic region which is

involved in the kinase activity also appears to be highly conserved in insulin receptors from other species, since antibodies to this site recognize insulin receptors from frogs, chickens, and mice with high affinity (Morgan et al., 1986a). These results suggest that the kinase domain may be important in the function of these receptors.

In summary, the present work indicates that the receptors for IGF-I and insulin are highly homologous. Similarities in subunit structure, kinase activity, proteolytic digestion fragments and antigenic structure domonstrate that the two receptors are closely related. A more detailed comparison of these two receptors must await the cloning and sequencing of a cDNA that encodes the IGF-I receptor.

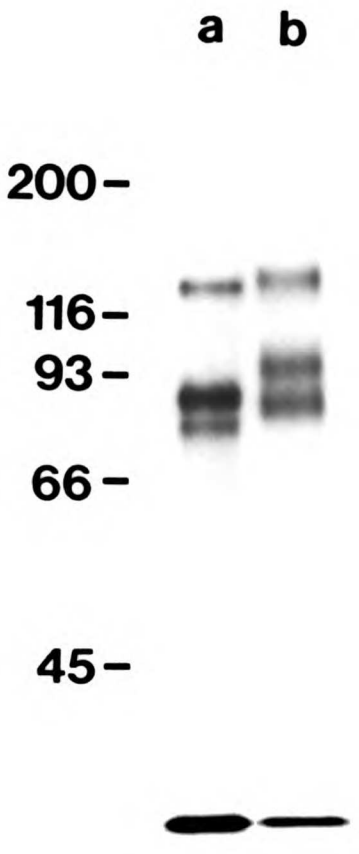

Figure 4.1. Subunit composition of insulin and IGF-I receptors.

Purified receptor preparations from rat liver (lane a) and purified IGF-I receptor from BRL-3A cells (lane b) were labeled with ^{125}I with Bolton Hunter reagent, reduced, and electrophoresed on a 7.5% polyacrylamide gel. An autoradiograph of the dried gel is shown. Molecular weights of protein standards (kDa) are shown.

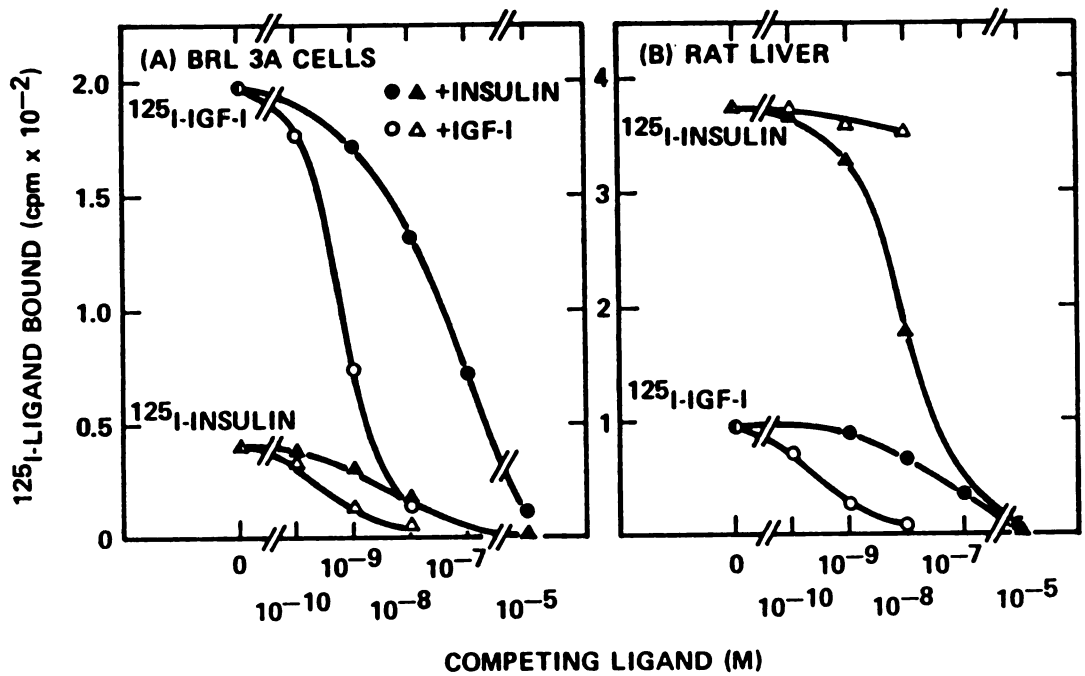

Figure 4.2. Binding of IGF-I and insulin to purified receptor preparations. Purified IGF-I receptor from BRL-3A cells (panel A) or insulin receptor from rat liver (panel B) were incubated with 0.8 nM ^{125}I-insulin (triangles) or 0.4 nM ^{125}I-IGF-I (circles) in the presence of indicated concentrations of unlabeled insulin (closed symbols) or IGF-I (open symbols). Bound radioactivity was measured as described in Experimental Procedures.

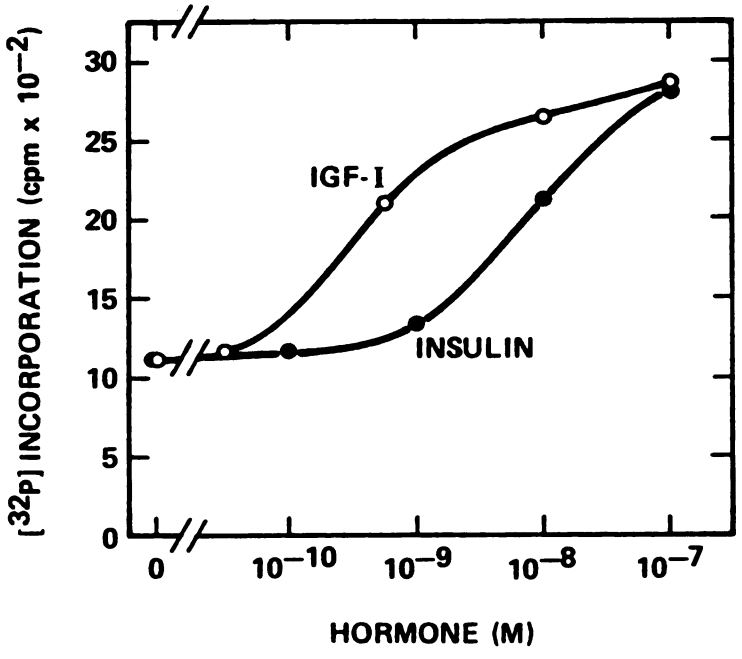

Figure 4.3. Stimulation of BRL-3A IGF-I receptor autophosphorylation by IGF-I and insulin.

Purified IGF-I receptor was incubated with Mn^{++}, [γ-^{32}P]ATP, and the indicated concentrations of IGF-I (○) or insulin (●). Reaction products were analyzed by electrophoresis, and the 85-94 kDa β-subunit bands were excised and counted by liquid scintillation. Values are means of duplicates, and similar results were obtained in four separate experiments.

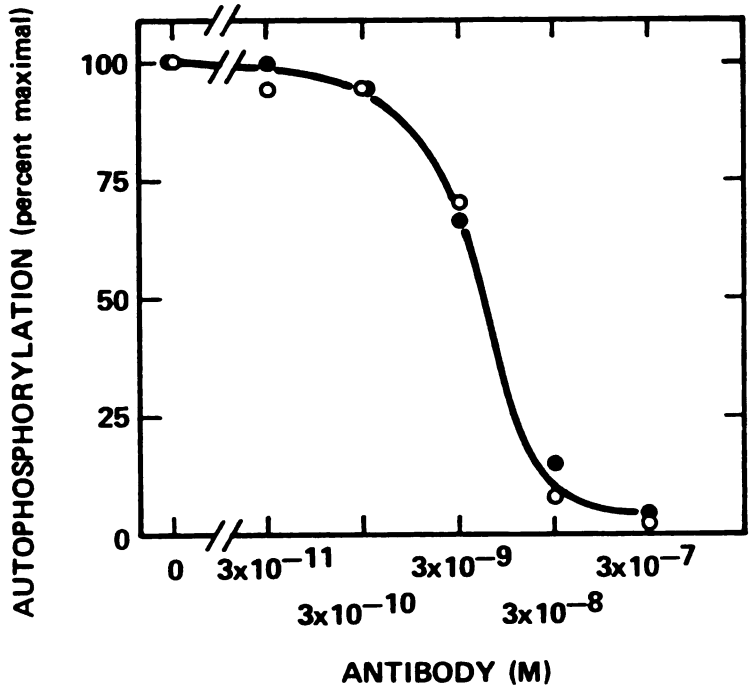

Figure 4.4. Inhibition of receptor phosphorylation by monoclonal antibody 17A3.

Either purified IGF-I receptor (5-10 ng) from BRL-3A cells (o) or rat liver insulin receptor (•) was incubated 60 min with Mn^{++}, the indicated concentration of antibody 17A3, and either IGF-I (30 nM, o) or insulin (30 nM, •). [γ-^{32}P]ATP was then added for an additional 60 min, and reaction products were analyzed by electrophoresis. The β-subunit bands were excised from the dried gel and counted by liquid scintillation. 100% values were 1888 cpm (o) and 527 cpm (•). Values are means of duplicates.

Figure 4.5. Protease mapping of receptor subunits.

Purified and ^{125}I-labeled insulin receptor from rat liver (lanes a-f) or IGF-I receptor from BRL-3A cells (lanes g-l) was denatured, reduced, and subjected to polyacrylamide gel electrophoresis. Separated α (top panel) and β (bottom panel) subunits were then eluted from gel slices and treated with the following concentrations of *Staphylococcus aureus* V8 protease: 0 (lanes a, g); 0.03 μg/ml (lanes b, h); 0.3 μg/ml (lanes c, i); 3 μg/ml (lanes d, j); 30 μg/ml (lanes e, k); 300 μg/ml (lanes f, l). Proteolyzed subunits were then electrophoresed on 15% polyacrylamide gels; autoradiographs of dried gels are shown. Molecular weights (kDa) of protein standards are shown.

CHAPTER 5

Acute Insulin Action Requires Insulin Receptor Kinase Activity: Introduction of an Inhibitory Monoclonal Antibody into Mammalian Cells Blocks the Rapid Effects of Insulin

Modified from a manuscript submitted for publication by:

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In the previous chapter, antibodies against the β_2 region of the insulin receptor kinase were found to inhibit the kinase activities of both the insulin and IGF-I receptors with equal potency. In the present chapter, as in Chapter 2, the ability of the β_2 antibodies to inhibit kinase activity was used to answer a fundamental question in this dissertation: what is the role of the kinase in insulin action? In Chapter 2, a long-term insulin effect in frog oocytes was found to be inhibited by the antibody. In the present chapter, these studies have been extended to demonstrate that the kinase is also involved in various rapid insulin effects in a variety of mammalian cell types.

SUMMARY

The role of the insulin receptor tyrosine kinase in various rapid insulin effects was studied by injecting four different cell types (by osmotic lysis of pinosomes) with a monoclonal antibody which specifically inhibits the kinase activity of the insulin receptor and the closely related IGF-I receptor. Injection of this inhibitory antibody resulted in a decreased ability of insulin to stimulate (i) the uptake of 2-deoxyglucose in Chinese hamster ovary cells and freshly isolated rat adipocytes; (ii) ribosomal protein S6 phosphorylation in CHO cells; and (iii) glycogen synthesis in the human hepatoma cell line HepG2. The ability of insulin, IGF-I, and IGF-II to stimulate glucose uptake in TA1 adipocytes was also inhibited. Studies with CHO cells demonstrated that these effects of the inhibitory antibody were specific, since (i) there was no change in phorbol ester-stimulated glucose uptake, and (ii) injection of a non-inhibiting antibody to the kinase had no effect on insulin action. These studies indicate that the tyrosine kinase activity of the insulin receptor is important in mediating several rapid insulin effects.

INTRODUCTION

The family of tyrosine-specific protein kinases includes a number of viral oncogene products and the receptors for several growth factors (epidermal growth factor, EGF; platelet-derived growth factor, PDGF; and insulin-like growth factor-I, IGF-I; Heldin and Westermark, 1984; Hunter and Cooper, 1985). Another member of this family is the insulin receptor, which possesses a tyrosine kinase in the cytoplasmic domain of its β subunit (Ullrich et al., 1985; Ebina et al., 1985b). The autophosphorylation and activation of this kinase are brought about by the binding of insulin to the extracellular domain of the receptor (for reviews see Czech, 1985; Kahn, 1985). Since many of insulin's actions are known to involve changes in protein phosphorylation (Avruch et al., 1982; Denton et al., 1981), the discovery of this kinase appeared at first to provide the elusive key to the molecular mechanism of insulin action. However, efforts to establish a role for the kinase in insulin action have not been entirely successful. Although several lines of indirect evidence implicate the kinase in the various short- to long-term actions of insulin (Kahn, 1985), there is still some question regarding its exact function, particularly in the rapid effects of insulin.

Recently, it has become possible to directly assess the physiological role of the receptor kinase by devising methods to block kinase activity in the intact cell. Two approaches have been used in these efforts. The first is based on the expression of normal and mutated insulin receptors in cell lines possessing few endogenous receptors. Recently, Ebina et al. (1985a) and Ellis et al. (1986) successfully expressed high levels of functional human insulin receptors in Chinese hamster ovary (CHO) cells. Then, in order to obtain kinase-deficient mutants, Ellis et al. (1986) developed CHO cells which expressed receptors in which important autophosphorylation sites of the kinase (tyrosines 1162 and 1163) were changed to phenylalanine by site-directed mutagenesis. The kinase activity of these mutated

receptors was greatly reduced both *in vitro* and *in vivo*. This loss of kinase activity was accompanied by a similar loss in the ability of insulin to stimulate membrane glucose transport, suggesting that this rapid insulin effect is mediated by the receptor kinase.

Methods involving expression of mutated receptors have a major limitation in that they cannot be readily applied to a broad range of cell types. Generally, the technique is limited to possibly abnormal cell lines possessing few insulin receptors, and insulin action in primary cells cannot be analyzed. Thus, we have recently devised a second method for inhibiting receptor kinase activity in the intact cell, which can be used in most cell types and thus circumvents the limitations of the first approach. A panel of monoclonal antibodies to the insulin receptor kinase was developed (Morgan et al., 1986a), and several of these antibodies were found to be extremely potent inhibitors of the receptor kinase activity. These inhibitory antibodies, known as the β_2 antibodies, bind to an antigenic region comprised in part by the tyrosine 1162/1163 autophosphorylation sites (Ellis et al., 1986). Thus, it was possible to inhibit receptor kinase activity in the intact cell by introducing these antibodies into the cytoplasm of insulin target cells. Kinase inhibition by this method is specific, since the β_2 antibodies do not recognize several related tyrosine kinases, including the EGF receptor, the PDGF receptor, and the protein products of the viral oncogenes *v-fms* and *v-ros* (Morgan et al., 1986a). These antibodies do, however, recognize and inhibit the tyrosine kinase activity of the IGF-I receptor, whose subunit structure and tyrosine kinase characteristics are remarkably similar to those of the insulin receptor (Rechler and Nissley, 1985; Sasaki et al., 1985; LeBon et al., 1986; Morgan et al., 1986b). Thus, since insulin binds with low affinity to the IGF-I receptor (Rechler and Nissley, 1985), injection of the β_2 antibodies will also affect actions of insulin which result from its interaction at high doses with the IGF-I receptor.

In previous studies (Morgan et al., 1986a), direct microinjection of one of the inhibitory antibodies (antibody 17A3) into *Xenopus* oocytes was used to demonstrate that inhibition of receptor kinase activity results in an inhibition of insulin's ability to stimulate

oocyte maturation. The injection of a non-inhibiting antibody to the receptor kinase had no effect. Thus, these results supported a role for the kinase in this long-term effect of insulin.

In order to probe the function of the kinase in the rapid effects of insulin, a method more practical than microinjection was needed for efficiently introducing the inhibitory antibody into large numbers of various mammalian cell types. Okada & Rechsteiner (1982) have recently developed a simple and versatile method which takes advantage of the process of fluid-phase pinocytosis which normally occurs in most cells. In this procedure, cells are first exposed to a hypertonic solution containing the desired macromolecule. Pinocytosis of this solution results in the formation of hypertonic vesicles, which can be selectively lysed by brief exposure of the cells to mildly hypotonic medium. The macromolecule is thus released into the cytosol, while cell viability is largely unaffected.

In the present studies this technique has been used to introduce the inhibitory antibody into the cytoplasm of several cultured cell lines, as well as primary rat adipocytes. Several rapid and intermediate actions of insulin and IGF-I were found to be inhibited by the antibody, providing direct evidence that the receptor kinase mediates the acute actions of insulin in classical insulin target cells.

EXPERIMENTAL PROCEDURES

Materials

Purified pork insulin was purchased from Elanco; human IGF-I (a product of recombinant DNA technology) was a generous gift of J. Merryweather, Chiron Corp.; purified rat IGF-II (MSA III-2) was a generous gift of S. Peter Nissley, National Institutes of Health; [^{32}P]H_3PO_4 (carrier free), 2-deoxy-D-[1,2-^{3}H]glucose (53 Ci/mmol), and D-[6-^{3}H]glucose (25 Ci/mmol) are from ICN; phloretin, glycogen, human γ-globulins (Cohn fraction II), phorbol-12-myristate-13-acetate (PMA), and silicone oil (dimethylpolysiloxane, 100 centistokes) are from Sigma; poly(ethylene glycol) (PEG) 1000

is from Baker, bovine serum albumin (BSA) is from Armour Pharmaceutical; collagenase is from Worthington; normal mouse immunoglobulin (IgG) is from Cappel; and Matrigel basement membrane is from Collaborative Research. Protein A-purified monoclonal antibodies 17A3 and 1G2 have been described previously (Morgan et al., 1986a, Morgan and Roth, 1986).

Preparation of Hypertonic Antibody Solutions

Normal IgG (human or mouse) or purified monoclonal anti-receptor antibody (17A3 or 1G2) were dialyzed against 50 mM Hepes, pH 6.9, 150 mM NaCl and then concentrated in a Centricon 30 concentrator (Amicon) to a final concentration of ≈50 mg/ml in a volume of 280 µl. The addition of sucrose (68 mg) and 50% PEG 1000 (80 µl) to this antibody concentrate resulted in a final volume of ≈0.4 ml hypertonic antibody solution with approximate final concentrations of 0.5 M sucrose, 10% PEG 1000, 35 mM Hepes, pH 6.9, 105 mM NaCl, and 30 mg/ml antibody.

Antibody Loading of CHO, HepG2, and TA1 Cell Lines

In order to minimize the amount of hypertonic antibody solutions required, the method of Okada & Rechsteiner (1982) was modified for use with cells in suspension. In most experiments, as recommended by Wright (1984), a minimum of 0.4 ml hypertonic solution was used for the treatment of 10^7 cells; for experiments with larger cell numbers (protein S6 phosphorylation), larger volumes (2 ml) were prepared.

Trypsinized cells (10^7 per tube) were washed once with PBS (20 mM $NaPO_4$, pH 7.4, 150 mM NaCl) and centrifuged 7 min at 700 x g. After carefully aspirating all traces of the supernatant, cell pellets were resuspended vigorously in 0.4 ml of warm hypertonic antibody solution. After 10 min at 37°C with occasional shaking, the hypertonic cell suspension was diluted with 10 ml 37°C hypotonic medium (60% serum-free Ham's F-12/40% H_2O). After 2 min at 37°C, the cells were centrifuged, the supernatant was

removed (for later repurification of unused antibody), and the cells were then resuspended in culture medium and treated as required for each bioassay (below).

Glucose Uptake by CHO Cells

CHO cells were loaded as described above with the desired antibody and resuspended in normal culture medium (Ham's F-12/10% fetal calf serum, FCS). Cells were then plated in 24-well tissue culture plates (4 X 10^5 cells per 16 mm well), and incubated in 5% CO_2 at 37°C for 12 h, the time required for the cells to attach and form confluent monolayers (Best inhibitory results were obtained when bioasssays were performed within 16-20 h of injection, suggesting that antibody degradation inside the cell may become a problem after long periods). After 12 h, medium was changed to fresh medium containing 10 mM 2-deoxyglucose, which lowers basal glucose uptake and thus enhances insulin responsiveness. After 4 h at 37°C, wells were washed twice with warm DB/BSA (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.1 % BSA, pH 7.4) and incubated 30 min at 37°C with 0.5 ml of the same buffer. The desired concentration of insulin or PMA was added for 15 min at 37°C; 0.1 mM 2-deoxy-D-[1,2-^{3}H]glucose (0.4 μCi/ml) was added for the last 10 min of this incubation. Cells were washed twice with ice-cold DB containing 100 μM phloretin, solubilized with 0.03% sodium dodecyl sulfate, and counted by liquid scintillation. Protein assays of cell lysates confirmed that there were no differences in cell number between groups loaded with different antibodies. In some experiments, extra wells of injected cells were plated and tested 12 h later for their ability to bind ^{125}I-labeled insulin, as described (Ellis et al., 1986).

Ribosomal Protein S6 Phosphorylation in CHO Cells

CHO cells (2 X 10^7 cells per antibody) were loaded with 2 ml hypertonic solution, resuspended in normal culture medium, and distributed into 100 mm tissue culture plates at

high density (4 X 10^6 cells/dish). After 12 h at 37°C, dishes were washed twice with KRBB/BSA (107 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 7 mM $NaHCO_3$, 10 mM glucose, 0.1% BSA, pH 7.4) and then incubated 3 h at 37°C with 4 ml KRBB/BSA containing 200 μCi [^{32}P]H_3PO_4. Cells were then treated with indicated concentrations of insulin for 15 min at 37°C, washed twice with ice-cold Tris-buffered saline and solubilized with 3 ml ice-cold 50 mM Hepes, 5 mM KCl, 5 mM $MgCl_2$, 50 mM NaF, 40 μM EDTA, 1% Triton X-100, 1% sodium deoxycholate, pH 7.5. This lysate was centrifuged at 10,000xg for 10 min and the supernatant was layered over 3 ml of 1.6 M sucrose, 50 mM Hepes, 5 mM $MgCl_2$, 0.5 M KCl, 40 μM EDTA, pH 7.5 and centrifuged at 140,000xg for 2 h at 4°C. Ribosomal pellets were washed and analyzed by electrophoresis on 12.5% polyacrylamide gels.

Glycogen Synthesis by HepG2 Cells

Human hepatoma cells (HepG2), loaded with the desired antibody, were resuspended in culture medium (Eagle's MEM/Earle's salts/10% FCS) and plated onto 24-well culture plates (4 X 10^5 cells/well) coated with 0.1 ml/well of Matrigel basement membrane. After 14 h at 37°C, wells were washed twice with warm DB containing 1% BSA and 1 mM glucose, and then incubated 2 h at 37°C with 0.5 ml of the same buffer containing 3 μCi D-[6-^{3}H]glucose and the desired concentration of insulin. Wells were washed twice with ice-cold PBS, and cells were then solubilized by the addition of 0.5 ml 30% KOH containing 3 mg/ml glycogen. After vigorous shaking for 30 min at 24°C, contents of the wells were transferred to glass tubes, wells were rinsed with another 0.25 ml KOH/glycogen, and the solutions were boiled 30 min. Tubes were then cooled to 4°C, and 1.7 ml ice-cold ethanol was added. After 16 h incubation at 4°C, tubes were centrifuged (2000xg, 30 min, 4°C) and the supernatant discarded. Pellets were dissolved in 0.75 ml H_2O, and then 1.5 ml ice-cold ethanol was added. After 2 h at 4°C, precipitates were once

again pelleted by centrifugation, supernatants were discarded, and the pellets dissolved in 0.2 ml H_2O and counted by liquid scintillation.

Glucose Uptake by TA1 Adipocytes

Confluent monolayers of TA1 cells, growing in Eagle's MEM/Earle's salts/10% FCS, were induced to differentiate into adipocytes by three days treatment with 0.1 mM indomethacin (D.Knight and G. Ringold, pers. comm.). Medium was then replaced with normal culture medium and cells were used one or two days later. Cells were treated with 10 mM 2-deoxyglucose for 4 h, loaded with antibody as described above, and resuspended in serum-free medium containing 10 mM 2-deoxyglucose for 1 h at 37°C. Cells were then washed with DB/BSA, distributed into glass tubes (3 X 10^5 cells each), and incubated with the desired concentration of hormone for 30 min at 37°C. 2-deoxy-D-[1,2-^{3}H]glucose (0.1 mM, 0.4 μCi/ml) was then added for 10 min at 37°C, followed by termination of the reaction with ice-cold DB/100mM phloretin. After two washes with ice-cold DB/phloretin, cells were solubilized with 200 μl 0.03% sodium dodecyl sulfate and counted by liquid scintillation.

Antibody Loading and Glucose Uptake of Rat Adipocytes

Isolated adipocytes were obtained from epididymal fat pads of male rats (300 g) by standard techniques (Rodbell, 1964; Hoffman et al., 1984) with minor modifications: fat pads were minced and incubated 30 min at 37°C in FC buffer (131 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 10 mM Hepes, pH 7.4) containing 3% BSA, 10 mM glucose, and 1 mg/ml collagenase. After passage through nylon mesh, cells were gently washed 3 times with the same buffer lacking collagenase. Tubes containing approximately 0.3 ml packed cells were then washed once with hypertonic PEG/sucrose solution lacking antibody and then resuspended in 0.4 ml of the desired hypertonic antibody solution. After 10 min of gentle shaking at 37°C, 10 ml

hypotonic medium was added for 5 min at 37°C, after which the infranatant was removed. The cells were then resuspended in FC buffer containing 3% BSA and 10 mM 2-deoxyglucose and incubated 1 h at 37°C. Cells were then washed once with FC/BSA, resuspended in FC/BSA, and distributed into 12 polyethylene tubes (250 µl/tube) in the presence of the desired concentration of insulin. Labeled 2-deoxyglucose uptake was then measured as described above for TA1 cells, except that reactions were terminated by centrifuging the cells through 100 µl silicone oil layers in 400 µl microfuge tubes. The lower aqueous layer was cut off and discarded, and the upper portions of the tubes, containing the separated cells, were counted in liquid scintillation fluid.

RESULTS

Glucose Uptake by CHO Cells

Our initial studies involved the effect of kinase inhibition on one of the most well characterized and rapid responses to insulin: the stimulation of membrane glucose transport (Cushman et al., 1984; Kono, 1984). CHO cells were chosen for these experiments since (i) they exhibit a sensitive 2-3-fold increase in glucose uptake in response to insulin (Ebina et al., 1985a), and (ii) Ghosh et al. (1984) have found that CHO cells can be successfully injected by the osmotic pinosome lysis technique without adversely affecting cell viability.

In order to determine if the inhibition of insulin receptor kinase activity *in vivo* affects insulin responsiveness, CHO cells were loaded with either normal immunoglobulin (IgG) or the inhibitory monoclonal antibody 17A3. In normal CHO cells and in cells injected with control IgG (Figure 5.1A), 15 min exposure to insulin resulted in a dose-dependent enhancement of the uptake of labeled 2-deoxyglucose: a two-fold stimulation of uptake occurred at 3 nM insulin. In cells loaded with the inhibitory antibody 17A3, both the sensitivity to insulin and the maximum insulin response were reduced. At low insulin concentrations (3 nM), the response was inhibited 80%. In addition, basal glucose uptake

(in the absence of added insulin) was 20% lower in cells loaded with antibody 17A3. To determine whether the inhibitory effect of antibody 17A3 was specific, CHO cells were osmotically injected with monoclonal antibody 1G2, which also recognizes the cytoplasmic domain of the insulin receptor but has little effect on receptor autophosphorylation or receptor kinase activity (Morgan et al., 1986a; Morgan and Roth, 1986). Insulin-stimulated glucose uptake was only slightly inhibited (10% at 3 nM insulin) in cells loaded with this antibody (Figure 5.1A).

The injection of antibodies which bind to an internal receptor domain could induce receptor clustering and internalization, resulting in decreased receptor number and loss of insulin sensitivity. To test for this possibility, the binding of ^{125}I-labeled insulin was studied in additional wells of the same cells injected with antibodies in the above experiments. Cells were incubated with ^{125}I-insulin (50,000 cpm) for 16 h at 4°C, in the presence or absence of excess unlabeled insulin (see Experimental Procedures). Cells loaded with control IgG, antibody 1G2, and antibody 17A3 were found to specifically bind 141±27, 161±7, and 136±12 cpm of ^{125}I-insulin, respectively, indicating that no significant changes in receptor number had occurred.

The specificity of the inhibitory effects of antibody 17A3 was further tested in studies where glucose transport was stimulated by an agent which would be expected to act by a mechanism not involving the insulin receptor. Phorbol esters, such as phorbol-12-myristate-13-acetate (PMA), are known to stimulate glucose uptake in a variety of cell types (Driedger and Blumberg, 1977; Lee and Weinstein, 1979), possibly via the direct phosphorylation of the glucose transporter by protein kinase C (Witters et al., 1985). Thus, antibody 17A3 was tested for its ability to inhibit the response of CHO cells to phorbol esters. CHO cells, loaded as before with either control IgG or antibody 17A3, were treated for 15 min at 37°C with various doses of insulin or PMA (Figure 5.1B). As before, the insulin response was greatly reduced in cells loaded with antibody 17A3. In contrast, both the fold stimulation by PMA and the sensitivity to PMA were not affected by antibody

17A3. A slight downward shift of the dose-response curve for PMA was observed in cells loaded with antibody 17A3; this is presumably a consequence of the ability of 17A3 to decrease the basal glucose uptake in these cells, as mentioned above.

Ribosomal Protein S6 Phosphorylation in CHO Cells

To determine if another acute effect of insulin also involves the receptor kinase, we studied insulin-stimulated phosphorylation of ribosomal protein S6 (Thomas et al., 1982; Avruch et al., 1982). As before, CHO cells were osmotically injected with either control IgG or antibody 17A3. Twelve hours later, semi-confluent monolayers of injected cells were labeled for 3 h at 37°C with [^{32}P]orthophosphate and then treated with or without insulin for 15 min at 37°C. Polyacrylamide gel analyses of ribosomal preparations from these cells revealed that insulin greatly stimulated the phosphorylation of a protein of molecular weight M_r=34,000 (Figure 5.2A). Previous studies with a variety of cell types indicate that this protein is ribosomal protein S6 (Thomas et al., 1982; Avruch et al., 1982; Wool, 1979). In cells injected with control IgG, S6 phosphorylation was stimulated over 6-fold by insulin, and half-maximal stimulation occurred at 0.5 nM insulin. The insulin response was dramatically reduced in cells loaded with the inhibitory antibody 17A3: more than 100 nM insulin was required to give a 3-fold stimulation of S6 phosphorylation in these cells (Figure 5.2B).

Glycogen Synthesis by HepG2 Cells

In addition to studying rapid insulin effects, we wished to analyze the effect of kinase inhibition on the intermediate actions of insulin. The human hepatoma cell line HepG2 (Knowles et al., 1980) was chosen for this purpose, since this cell line is known to exhibit insulin-stimulated glycogen synthesis, a major intermediate insulin effect (Verspohl et al., 1984; Podskalny et al., 1985).

HepG2 monolayers, loaded as above with either control IgG or the inhibitory antibody 17A3, were tested for their ability to incorporate labeled glucose into glycogen in response to insulin (Figure 5.3). Glycogen synthesis was stimulated about 2-fold in control cells, with half-maximal stimulation occurring at about 0.5 nM insulin. Cells injected with the inhibitory antibody 17A3 exhibited an 80% reduction in the insulin response: even at maximal insulin concentrations (300 nM), glycogen synthesis was stimulated only 20% (Figure 5.3).

Glucose Uptake by Isolated Rat Adipocytes

A classic target cell in studies of insulin action is the rat adipocyte, which exhibits a major increase in glucose uptake in response to low doses of insulin (Cushman et al., 1984; Kono, 1984). Thus, to definitively establish the role of the kinase in insulin's rapid effects, freshly isolated rat adipocytes were loaded with control IgG or antibody 17A3 by osmotic lysis of pinosomes. Despite their fragility, a majority of the adipocytes remained intact after the treatment and could be analyzed for their ability to transport glucose in response to insulin (Figure 5.4). Control cells exhibited a sensitive 3-4-fold insulin response. The injection of antibody 17A3 caused an even greater inhibition of insulin responsiveness in these cells than was observed in CHO cells: the response was 90-95% inhibited at moderate insulin concentrations (0.3 to 3 nM).

Insulin and IGF Actions on TA1 Adipocytes

Since antibody 17A3 also inhibits IGF-I receptor kinase activity (Morgan et al., 1986b), it was possible to demonstrate a role for the kinase in the actions of IGF-I. Similarly, our studies could be used to clarify the actions of a related polypeptide, IGF-II, which binds mainly to its own receptor but also binds with moderate affinity to the IGF-I receptor (Rechler and Nissley, 1985). Antibody 17A3 does not recognize the IGF-II receptor,

which is structurally quite distinct from the insulin and IGF-I receptors and does not appear to possess an intrinsic tyrosine kinase activity (Corvera et al., 1986).

These experiments were performed with the TA1 cell line, a cell line which, like 3T3-L1 cells, undergoes a differentiation process from a fibroblast to an adipocyte phenotype (Chapman et al., 1984). For the present studies we used TA1 adipocytes, which exhibit rapid glucose uptake responses to insulin, IGF-I, and IGF-II (Shimizu et al., 1986). Over 2-fold increases in glucose uptake were seen in control IgG-injected cells treated with each of the three polypeptides (Figure 5.5); half-maximal stimulation occurred at approximately 3 nM insulin, 0.3 nM IGF-I, and 1 nM IGF-II. The introduction of antibody 17A3 into these cells resulted in an inhibition of the response to all three factors: the response was inhibited 78%, 58%, and 69% in cells treated with 3 nM concentrations of insulin, IGF-I, and IGF-II, respectively (Figure 5.5).

DISCUSSION

Effects of Receptor Kinase Inhibition on Acute Insulin Action

Tyrosine kinase activity is a property shared by several growth factor receptors and transforming proteins (Hunter and Cooper, 1985), implying that tyrosine phosphorylation is fundamentally involved in cellular growth and metabolism. Thus, it is reasonable to hypothesize that the actions of insulin (a major regulator of these cellular processes) are mediated by the tyrosine kinase activity of its receptor. The purpose of the present work was to test this hypothesis by analyzing insulin action after blocking the receptor kinase activity *in vivo*. Osmotic lysis of pinosomes (Okada and Rechsteiner, 1982) was used to introduce into the cell cytoplasm a monoclonal antibody which inhibits insulin (and IGF-I) receptor kinase activity by binding to a highly conserved autophosphorylation site made up in part by tyrosines 1162 and 1163 of the human insulin receptor (Ellis et al., 1986).

The present results support a role for the kinase in insulin action. The inhibition of receptor kinase activity in four different mammalian cell types was found to inhibit three important rapid and intermediate insulin actions. To begin with, insulin-stimulated glucose uptake was found to be inhibited by the injection of antibody 17A3 into two cell lines, the CHO and TA1 lines, as well as primary rat adipocytes. In CHO cells, the inhibitory effect of the antibody was specific, since (i) a non-inhibiting antibody to the kinase (1G2) had no significant effect on insulin action, and (ii) the stimulation of glucose uptake by an unrelated agent (PMA) was not affected by the anti-receptor antibody.

The actions of the insulin-like growth factors on glucose uptake were also examined in these studies. Since the inhibitory antibody 17A3 also recognizes the IGF-I receptor, we could demonstrate that the effect of IGF-I on glucose uptake in the TA1 cell also involves its receptor kinase. The actions of IGF-II have also been clarified in these experiments. IGF-II can bind with moderate affinity to the IGF-I receptor as well as its own receptor, and so it is sometimes difficult to determine which of the two IGF receptors mediates IGF-II action. The antibody injection technique provides a useful new method for determining the receptor type through which IGF-II is acting. Since the inhibitory antibody does not recognize the IGF-II receptor, then only IGF-II effects which are mediated by the IGF-I receptor should be inhibited by antibody 17A3. This was indeed the case in the present work, indicating that the effect of IGF-II on glucose uptake in these cells is largely mediated by the IGF-I receptor kinase.

Some instructive differences were observed between the three cell types studied in these glucose uptake experiments. First, antibody 17A3 injection caused a small decrease in basal glucose uptake in the CHO cells but not in TA1 cells or rat adipocytes. Since the CHO cells were growing in serum-containing medium until 30 min before the assay, it is possible that some insulin or IGF-I was present in these experiments due to incomplete removal of these serum factors. In contrast, TA1 cells and rat adipocytes were subjected to several buffer changes and over 2 hours of serum-free conditions prior to assay (including the time

involved in injection); thus, it is unlikely that any serum factors were present. Presumably for similar reasons, no antibody inhibition of basal activity was seen in the experiments involving S6 phosphorylation or glycogen synthesis (Figures 5.2 and 5.3).

Studies of glucose uptake in the three cell types also revealed differences in the degree of inhibition by antibody 17A3. Inhibition of insulin action was clearly more extensive in the rat adipocyte (Figure 5.4). This observation may be partly explained by differences in the normal rate of pinocytosis in different cell types (Okada and Rechsteiner, 1982). A more likely explanation is simply that the cytoplasmic volume of the rat fat cell, in relation to cell surface area, is extremely low; thus, the cytoplasmic concentration of antibody after injection should be higher in these cells.

Our studies of ribosomal protein S6 phosphorylation in CHO cells also revealed differences in the degree of response inhibition by antibody 17A3. Even when CHO cells from the same injection experiment were analyzed, this antibody appeared to have a greater inhibitory effect on the stimulation of S6 phosphorylation (Figure 5.2) than on the stimulation of glucose uptake (Figure 5.1). This may be the result of differences in the experimental conditions of the two assays or may reflect differences in the molecular events underlying these responses. The less extensive inhibition of the glucose uptake response in CHO cells does not necessarily indicate that the kinase is less important in this response, since in rat fat cells an almost complete inhibition of the glucose uptake response was seen after kinase inhibition. Interestingly, the inhibition of insulin-stimulated glycogen synthesis was also particularly extensive in HepG2 cells injected with the inhibitory antibody.

Does the Receptor Tyrosine Kinase Mediate All of Insulin's Effects ?

Until recently, the evidence supporting a role for the insulin receptor kinase in the actions of insulin has been largely indirect, and there has been some question regarding whether the kinase is actually involved in all of insulin's actions. The major evidence against a role for the kinase has come from studies of patients' sera containing antibodies against the

insulin receptor. Two of these antisera have been found to mimic insulin's effects on glucose uptake in the isolated rat adipocyte without stimulating receptor kinase activity in cell-free preparations or receptor phosphorylation in the intact adipocyte (Simpson and Hedo, 1984; Zick et al., 1984a). These results have provided an interesting challenge to the notion that the kinase is important; however, they are somewhat difficult to interpret, for the following reasons. First, polyclonal antisera may contain antibodies to a variety of cell surface antigens, some of which may be capable of stimulating glucose uptake by a mechanism entirely independent of the insulin receptor (Pillion et al., 1980; Beachy and Czech, 1980). Second, monoclonal and polyclonal anti-receptor antibodies are known to induce receptor internalization (Simpson and Hedo, 1984; Roth et al., 1983b; Maron et al., 1984; Grunfeld, 1984; Taylor and Marcus-Samuels, 1984); therefore, the insulinomimetic activity of anti-receptor antibodies may result from a relocation of the receptor to some internal compartment where basal kinase activity is sufficient to bring about the relatively moderate agonistic effects seen with these antibodies. In support of this possibility, Ellis et al. (1986) have observed that the expression of high levels of insulin receptor in CHO cells results in an increase in basal glucose uptake. Third, half-maximal stimulation of receptor phosphorylation in adipocytes occurs at concentrations of insulin which are 10 to 100-fold higher than the amounts required for half-maximal stimulation of glucose uptake (Haring et al., 1982); thus, it is conceivable that antibodies induce increased glucose uptake at low levels of receptor occupancy, where changes in receptor phosphorylation cannot be detected. Similarly, subtle changes in receptor phosphorylation involving increases at one site and decreases at another would go undetected. In addition, kinase activity toward exogenous substrates may be stimulated by antibodies in the absence of a detectable change in phosphorylation of the receptor itself. Fourth, studies of antibody effects on receptor phosphorylation in cell-free systems may not reflect the situation in the intact cell. Finally, as has been suggested by others (Zick et al., 1984a; Kahn, 1985), anti-receptor antibodies and insulin may stimulate biological effects by different mechanisms; thus, insulin may

induce a conformational change in the receptor by stimulating phosphorylation, whereas the antibodies produce this change without affecting phosphorylation.

On the other hand, several lines of indirect evidence implicate the kinase in short- and long-term insulin action. First, receptor kinase activity is decreased in pathological and experimental states of insulin resistance (Grigorescu et al., 1984; Grunberger et al., 1984; Kadowaki et al., 1984; LeMarchand-Brustel et al., 1985; Arsenis and Livingston, 1986; Burant et al., 1986). Second, phorbol esters cause a parallel decrease in receptor kinase activity and insulin responsiveness of cells (Takayama et al., 1984). Third, various agents which mimic insulin's bioeffects on intact cells (lectins, trypsin, or vanadate) also stimulate the receptor kinase activity (Roth et al., 1983a; Tamura et al., 1983, 1984).

More direct evidence is now available which demonstrates that the receptor kinase mediates several biological effects of insulin in various different cell types. In previous work (Morgan et al., 1986a), microinjection of an antibody which inhibits receptor kinase activity was found to block insulin-stimulated frog oocyte maturation. In the present studies, introduction of this antibody into mammalian cells was found to inhibit insulin's ability to stimulate glucose uptake, glycogen synthesis, and ribosomal protein S6 phosphorylation. It is possible that in addition to inhibiting the receptor kinase, this antibody affects other as yet undefined receptor functions which are crucial for insulin action. However, our conclusions are supported by previous studies demonstrating that mutated receptors deficient in kinase activity are also defective in their ability to transduce the signal for insulin-stimulated glucose uptake (Ellis et al., 1986).

Insulin's broad range of biological effects, including those studied in the present work, are mediated by a variety of post-receptor mechanisms. For example, the stimulation of glucose transport appears to involve a translocation of glucose transporter proteins from an intracellular site to the plasma membrane (Cushman et al., 1984; Kono, 1984). In addition, many of insulin's effects involve increased protein phosphorylation (as in the case of protein S6), whereas others involve dephosphorylation of specific proteins (as in the case

of insulin-stimulated glycogen synthetase activity)(Denton et al., 1981; Avruch et al., 1982). The finding that an inhibitory antibody to the kinase blocks responses which involve these different processes clearly implies that receptor kinase activity is a crucial signal which mediates a wide variety of insulin responses.

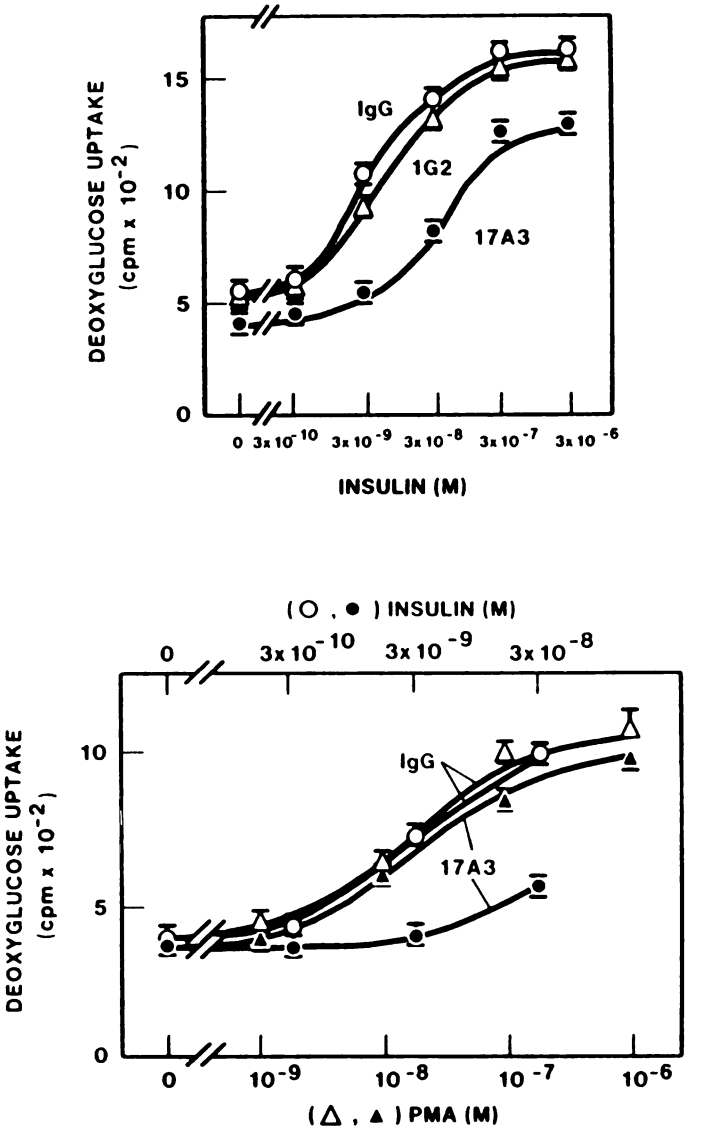

Figure 5.1. Effect of inhibitory antibody on glucose uptake by CHO cells. *Top panel*: Uptake of 2-deoxyglucose was measured in CHO cells injected with either normal IgG (o), monoclonal antibody 1G2 (Δ), or monoclonal antibody 17A3 (●), after 15 min incubation with the indicated concentrations of insulin. *Bottom panel*: Uptake of 2-deoxyglucose was measured in CHO cells injected with normal IgG (open symbols) or antibody 17A3 (closed symbols) after 15 min treatment with the indicated concentrations of either insulin (circles) or PMA (triangles). Values are means of triplicates ±S.D.

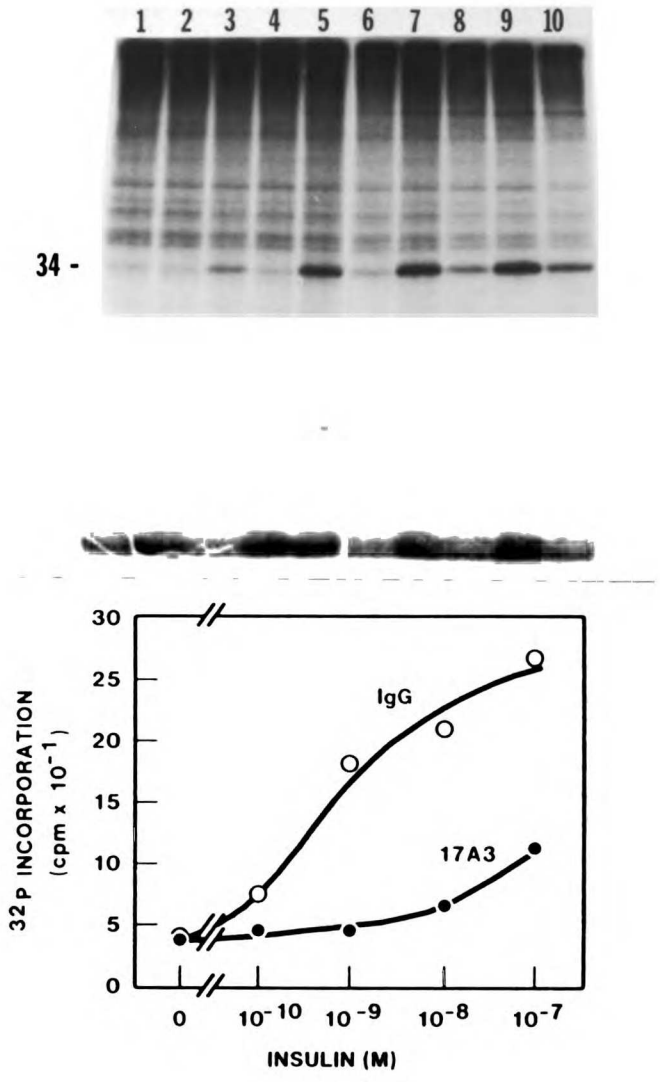

Figure 5.2. Effect of inhibitory antibody on insulin-stimulated phosphorylation of ribosomal protein S6 in CHO cells.

Top panel: Labeled phosphoproteins in a ribosomal preparation from CHO cells injected with either control IgG (lanes 1,3,5,7,9) or monoclonal antibody 17A3 (lanes 2,4,6,8,10) at the following insulin concentrations: 0 (lanes 1,2); 0.1 nM (lanes 3,4); 1.0 nM (lanes 5,6); 10 nM (lanes 7,8); 100 nM (lanes 9,10). *Bottom panel*: The 34 kDa band indicated in the top panel was excised from the gel and counted by liquid scintillation. Open and closed symbols represent CHO cells injected with control IgG and antibody 17A3, respectively. Similar results were obtained in two other experiments.

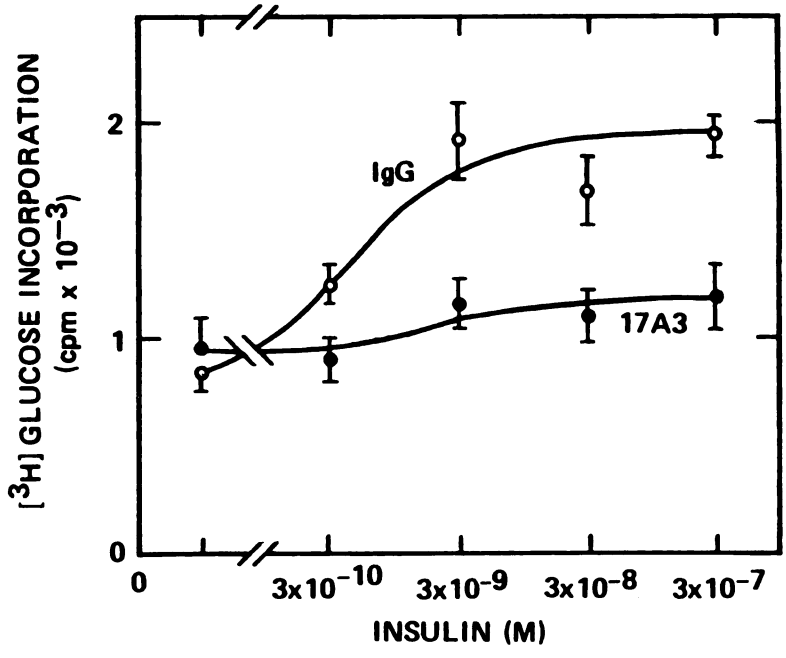

Figure 5.3. Effect of inhibitory antibody on insulin-stimulated glycogen synthesis in HepG2 cells.

Incorporation of labeled glucose into glycogen was measured in HepG2 cells injected with either control IgG (o) or antibody 17A3 (•) during a two hour treatment with the indicated concentrations of insulin. Values are means of triplicates ± S.D.

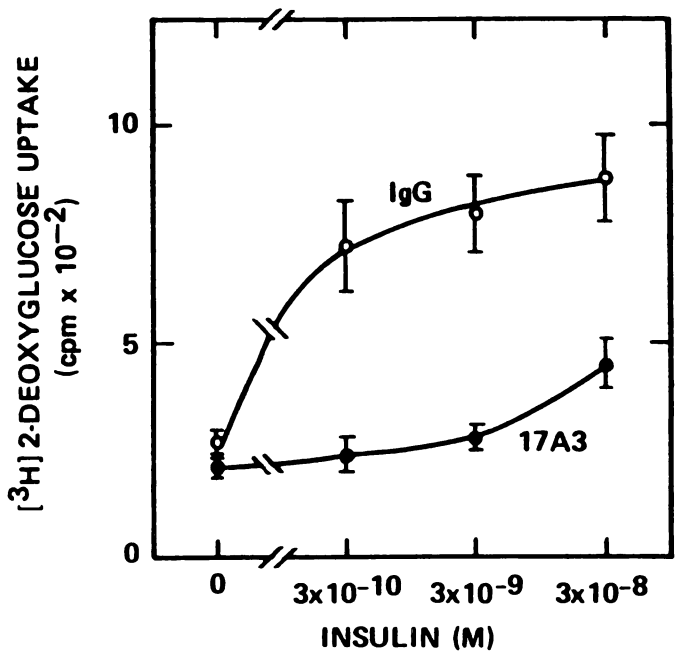

Figure 5.4. Effect of inhibitory antibody on insulin-stimulated glucose uptake by isolated rat adipocytes.

2-deoxyglucose uptake was measured in isolated rat adipocytes injected with either control IgG (○) or antibody 17A3 (●) after treatment with the indicated concentrations of insulin. Values are means of triplicates ± S.D.

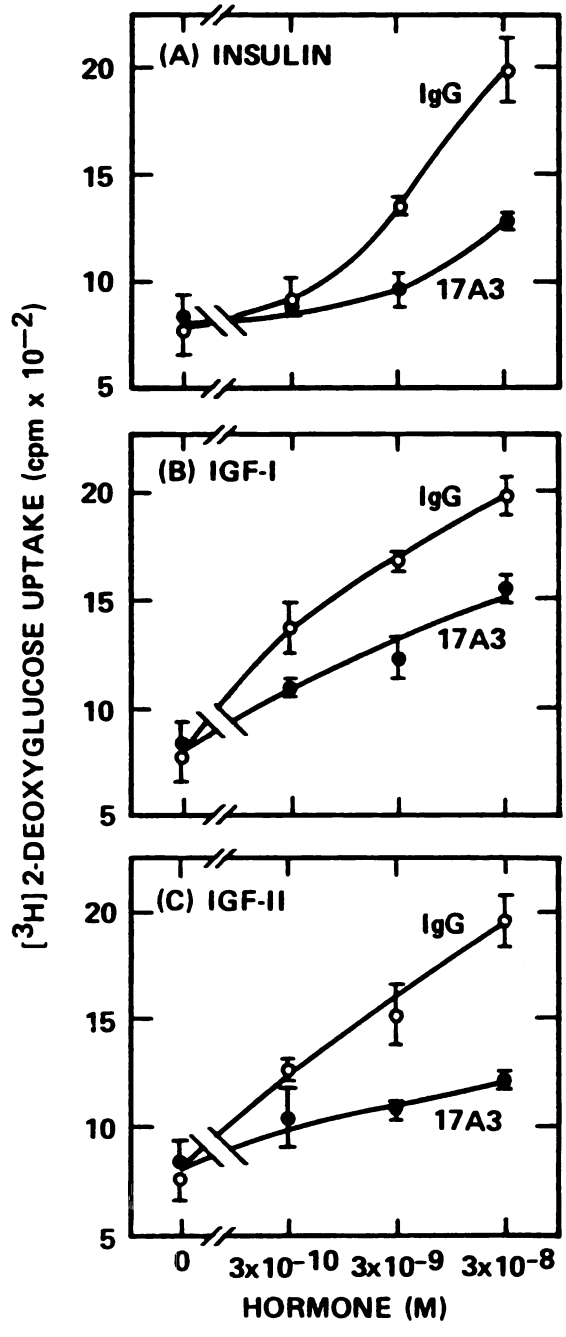

Figure 5.5. Effect of inhibitory antibody on stimulation of glucose uptake in TA1 cells by insulin and insulin-like growth factors.

2-deoxyglucose uptake was measured in TA1 adipocytes injected with either control IgG (o) or antibody 17A3 (•) after treatment with the indicated concentrations of insulin (panel A), IGF-I (panel B), or IGF-II (panel C). Values are means of triplicates ± S.D.

CONCLUSIONS

The results obtained in these experiments support a number of general conclusions beyond those already discussed in detail in the individual chapters. First, from a purely technical standpoint, it is reasonable to conclude from this work that monoclonal antibodies are exceptionally useful tools in studies of receptor structure and function. Initially, the antibodies developed in this work were employed for a variety of conventional purposes, including immunoprecipitation, receptor purification, and antigenic mapping. In further experiments, one particularly novel use for antibodies was developed: antibodies which inhibited the kinase activity of the receptor were introduced into various mammalian and frog cell types to clarify the role of the kinase in the actions of insulin. Although this method for studying enzyme function in the intact cell has not been commonly used in the past, the present work clearly illustrates its usefulness.

In addition, the following general conclusions can be made about the structure and function of the tyrosine-specific protein kinase in the cytoplasmic domain of the insulin receptor β subunit. This domain is relatively immunogenic, and contains at least four major antigenic regions, here named β_1 to β_4, which have been highly conserved during evolution. Antibodies to all of these regions influence kinase activity to some extent, but the β_2 antibodies are particularly potent kinase inhibitors. These highly inhibitory β_2 antibodies, unlike the antibodies to other regions, also recognize and inhibit the kinase activity of the purified IGF-I receptor. Taken together, these results suggest that the β_2 region is highly conserved in the two receptor types and is important for receptor function.

The development and characterization of the inhibitory β_2 antibodies made it possible to directly approach the original purpose of these studies, which was to clarify the role of the receptor-associated tyrosine kinase in the actions of insulin. The availability of these antibodies made it possible to inhibit receptor kinase activity *in vivo* by devising methods for introducing the inhibitory antibodies into the cytoplasm of various cell types. When the

antibodies were microinjected into frog oocytes, insulin's long-term effect on oocyte maturation was specifically inhibited. The introduction of the inhibitory antibody into the cytoplasm of Chinese hamster ovary cells, by osmotic lysis of pinosomes, specifically inhibited insulin's rapid stimulation of glucose uptake and ribosomal protein S6 phosphorylation. This antibody also dramatically inhibited insulin-stimulated glucose uptake in primary rat adipocytes, demonstrating that the inhibitory effect of this antibody could also be observed in a classical insulin target cell. In addition to inhibiting the effects of insulin, the antibody was also shown to inhibit insulin-like growth factor-stimulated glucose uptake by the TA1 mouse adipocyte line. Finally, the introduction of the inhibitory antibody into a human hepatoma cell line (HepG2) was found to inhibit insulin-stimulated glycogen synthesis, a well-known intermediate action of the hormone. The simplest interpretation of these results is that the rapid, intermediate, and long-term actions of insulin in a variety of cell types are mediated by the tyrosine kinase activity of its receptor.

The importance of the insulin receptor kinase in insulin action has also been revealed in recent studies of genetically engineered receptor mutants. The inhibition of kinase activity by mutation of crucial autophosphorylation sites was found to inhibit insulin-stimulated glucose uptake (Ellis et al., 1986). More recently (L. Ellis, pers. comm.), a truncated insulin receptor has been constructed which contains only the kinase domain, transmembrane sequence, and a small portion of the extracellular β subunit. This molecule possesses insulin-independent kinase activity and mediates a constitutively elevated glucose uptake. When the kinase activity of this truncated receptor is inhibited by mutagenesis of crucial autophosphorylation sites, glucose uptake is reduced to normal levels, further confirming the importance of the kinase in insulin action. Clearly, this approach has great potential: the generation of receptors possessing well-defined lesions in putative regulatory sites could provide a wealth of information regarding the function of various receptor domains. For example, the importance of receptor kinase activity could be further demonstrated by studies of receptors mutated at lysine 1030, which is believed to be

important in the ATP binding site of the kinase (Ebina et al., 1985b). In addition, future studies might involve the development of hybrid molecules, which could reveal the nature of transmembrane signalling and identify relationships between related tyrosine kinases. Such studies could include the construction of molecules containing external insulin receptor domains linked to kinase domains from related receptors (EGF, PDGF) or viral tyrosine kinases. Eventually, the development and characterization of these various mutant receptor molecules promises to provide major advances in our understanding of insulin receptor function.

However, the mechanism of insulin action beyond the receptor molecule remains largely unclear. Clearly, kinase activation may be the initial event from which various pathways of insulin action diverge. To confirm the importance of kinase activation, it will now be necessary to characterize the mechanisms by which the kinase initiates insulin action. As mentioned earlier, one of the potential mechanisms currently under investigation involves the phosphorylation by the kinase of specific protein substrates. Recently, the use of anti-phosphotyrosine antibodies has been useful for providing a glimpse of the structure of these substrates (see Introduction), but the functional characterization of these proteins will require their purification and sequencing. Since these substrates are present in exceedingly small amounts in the cell, their complete analysis will provide the same challenge as the original characterization of the insulin receptor itself. Eventually, the development of monoclonal antibodies to these substrates could lead to microinjection experiments similar to those described in the present work. Thus, using the injection of specific inhibitory antibodies, the role of a putative substrate could be clarified by inactivating the protein *in vivo*. Furthermore, receptor kinase autophosphorylation may mediate certain insulin actions in the absence of exogenous substrate phosphorylation. Thus, the autophosphorylation process may lead to the activation and/or detachment of receptor-associated effector molecules. Further studies will be necessary to test these various possibilities and incorporate them into existing knowledge of putative insulin

mediators and insulin-stimulated changes in protein phosphorylation. Clearly, the exploration of this labyrinth of molecular pathways will provide new insight into the complex mechanisms underlying the integration of cellular metabolism.

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