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

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ABSTRACT: 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 monoclonal 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 indicate 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.

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., 1982b; 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., 1982a). Second, specific proteolysis of the β subunit abolishes the kinase activity of the receptor without affecting insulin binding activity (Roth et al., 1983b; 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., 1985).

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., 1985).

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., 1985). 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., 1985). The remaining three antibodies were found to bind to the extracellular domain of the receptor, including the insulin binding site (Morgan et al., 1985). 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[1] (specific for γ

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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., 1983b; Morgan & Roth, 1985).

Methods

Insulin Receptor Purification and Labeling. Human placental insulin receptor (100 μg/placenta) was highly purified by sequential affinity chromatography on an anti-receptor antibody column and wheat germ agglutinin column as previously described (Roth et al., 1983). Coomassie blue stained polyacrylamide gels of these preparations yield only the 135- 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 (5×10^7 cells) were fused with Sp2/0 myeloma cells (5×10^7 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 (8×10^7 cells) were fused with Sp2/0 myeloma cells (4×10^7 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 (Fazekas & Scheidegger, 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 presence 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 first 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 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 preincubated 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 binding 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 hybridomas 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 in a γ counter and then subjected to reduced SDS–polyacrylamide gel electrophoresis. With the autoradiograph as a guide, the 95-kDa receptor β subunit band 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

[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; IGF, insulin-like growth factor; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IgG, immunoglobulin G; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

Table I: Properties of Monoclonal Antibodies to Insulin Receptor[a]

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	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	4E10	1580	40	40	μ
5D9	2220	370	2250	γ	16F5	920	10	50	μ
3D7	2690	2120	2110	γ	17A3	2120	1940	1980	γ
1C1	1960	60	80	μ	24D5	290	80	360	γ
1G2	1720	1090	1450	γ	28B7	2210	2360	1970	γ
7D5	3080	60	100	μ	17E5	2400	1470	2300	γ
8H2	2850	770	2790	γ	20D9	2060	540	2200	γ
13B4	2300	10	50	μ	21D3	2820	740	2580	γ
15B5	2760	1230	2680	γ	24B7	2560	940	2480	γ
16E8	2850	2460	2550	γ	2G7	3750	2160	3080	γ
17G6	1380	20	50	μ	3F10	2970	1400	3050	γ
19E3	1040	220	770	γ	17H5	1640	460	1390	γ
20B4	1390	820	1390	γ	19H9	1470	410	1240	γ
20G2	3290	1630	3070	γ	25D8	1200	180	30	μ
21C11	2290	860	2100	γ	27B2	1700	1310	1560	γ
28F2	3110	730	3100	γ	29E3	3650	2590	3890	γ
29B4	3450	2500	3510	γ	11B11	280	1890	40	γ
30D1	570	140	500	γ	25D4	1990	50	140	μ
30D3	2290	820	2300	γ					

[a] Microtiter wells were coated with rabbit anti-mouse IgG, protein A, or goat anti-mouse IgG (Fc fragment specific), as described under Methods. 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 monoclonal antibody to the insulin binding site. Results shown are representative of several experiments; interassay variability was less than 10%.

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 I). 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 I) or by iodination (data not shown).

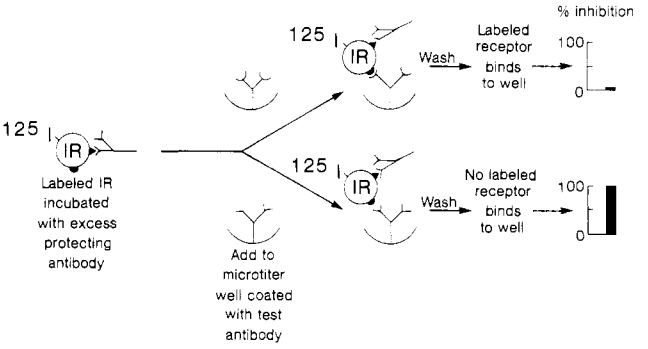

FIGURE 1: Method for mapping antigenic regions of the insulin receptor.

Eight of the 37 antibodies were not recognized by IgG-specific anti-mouse antibodies (Table I), 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 I).

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 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

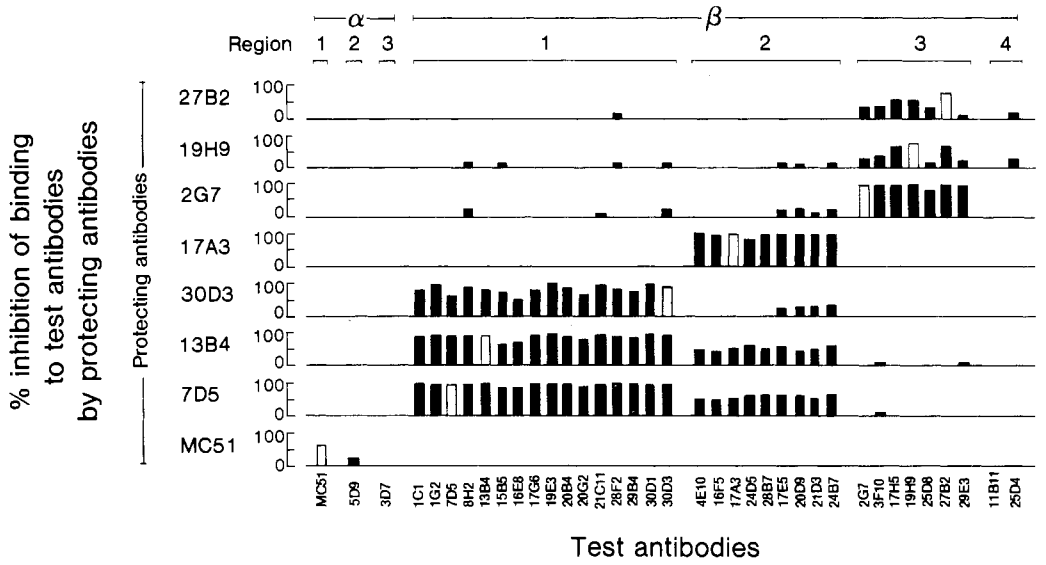

FIGURE 2: Antigenic regions of the insulin receptor. As outlined in Figure 1, labeled receptor was preincubated with the indicated protecting antibodies and then examined for binding to microtiter wells coated with the indicated test antibodies. The bars indicate the percent inhibition of receptor precipitation caused by the protecting antibody; this inhibition varied by less than 10% between experiments.

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 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 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 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 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_3 or β_1. 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 3A). The nine antibodies to β_2 all inhibited the autophosphorylation by more than 90%. Results with 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 4). The highly inhibitory β_2 antibodies all half-maximally precipitated the receptor at 2×10^{-9} M (Figure 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 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 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 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 autophosphorylation and histone phosphorylation occurred with the same antibody concentration and to the same maximal extent (Figure 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 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 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

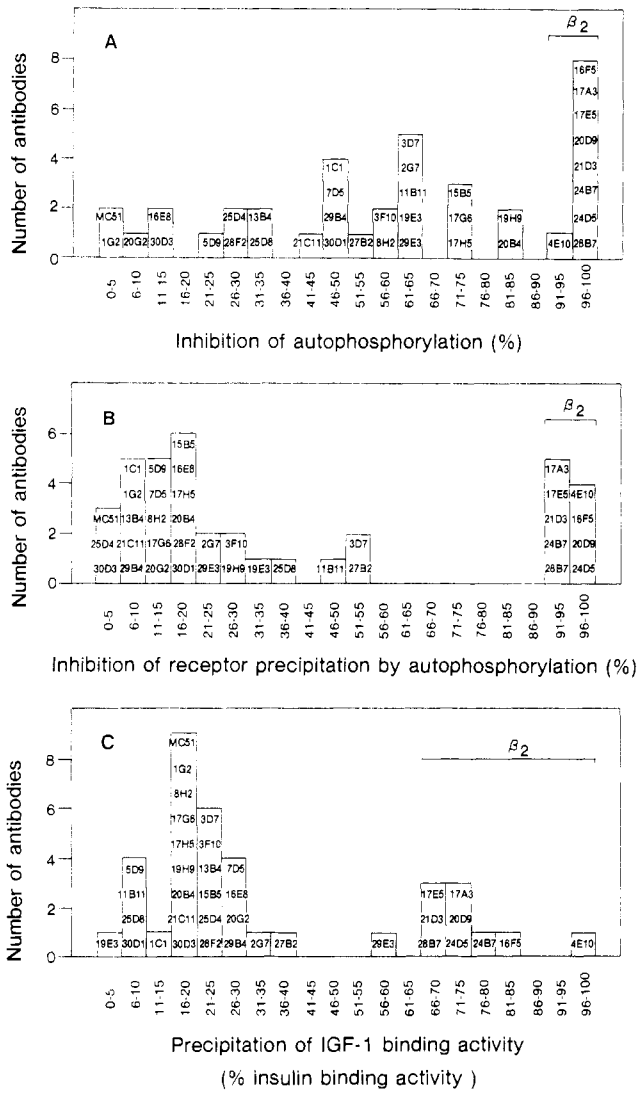

FIGURE 3: Mapping antigenic regions of the insulin receptor by functional assays. (A) Effect of antibodies on receptor autophosphorylation. The amount of ^{32}P incorporated into the β subunit of receptor precipitated by antibody-coated microtiter wells was determined. Values were normalized to the amount 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 Methods. 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 Methods. 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%.

under conditions where phosphorylation does not occur: for example, in the presence of ATP but without Mn^{2+} or in the presence of the nonhydrolyzable ATP analogue AMP-PNP and Mn^{2+} (Figure 6).

Precipitation of 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., 1983a; Kasuga et al., 1983) related to the insulin receptor, the monoclonal antibodies were tested for their ability to bind this receptor. The majority of the antibodies were

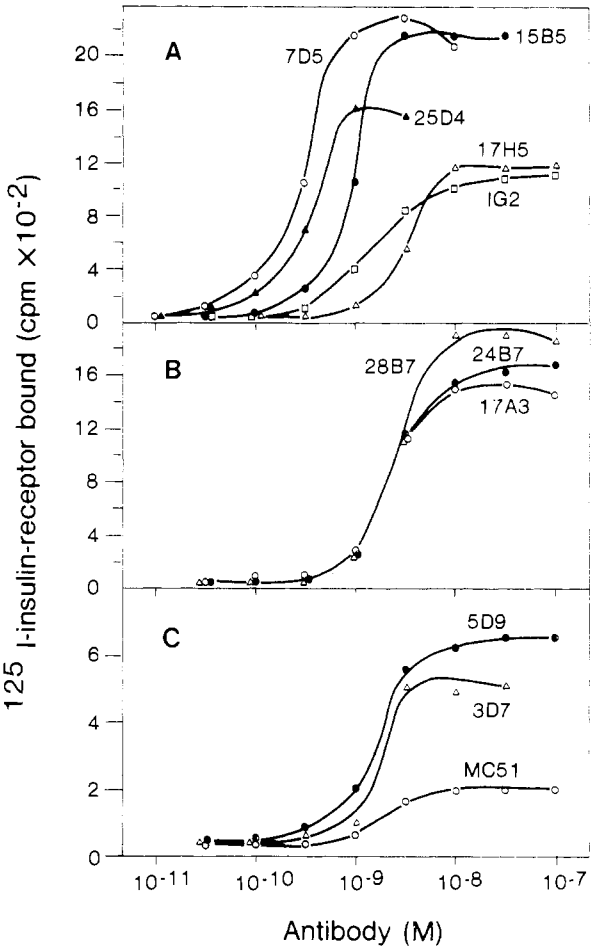

FIGURE 4: Precipitation of insulin receptor by different concentrations of several antibodies. Precipitation of cross-linked ^{125}I-insulin–receptor by microtiter wells coated with the indicated amounts of antibodies was performed with the plate precipitation assay described under Methods. ^{125}I-Insulin–receptor (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 (○), 15B5 (●), and 1G2 (□); β_3 antibody 17H5 (△); β_4 antibody 25D4 (▲). (B) β_2 antibodies 17A3 (○), 24B7 (●), and 28B7 (△). (C) α antibodies MC51 (○), 5D9 (●), and 3D7 (△). Similar results (±5%) were obtained in two separate experiments.

found to recognize the IGF-I receptor to only a limited extent (Figure 3C). However, the nine antibodies to β_2 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 activity 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 I, Figure 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., 1985).[2] Finally, affinity columns composed of several of these

[2] J. Beaudoin and R. Roth, unpublished results.

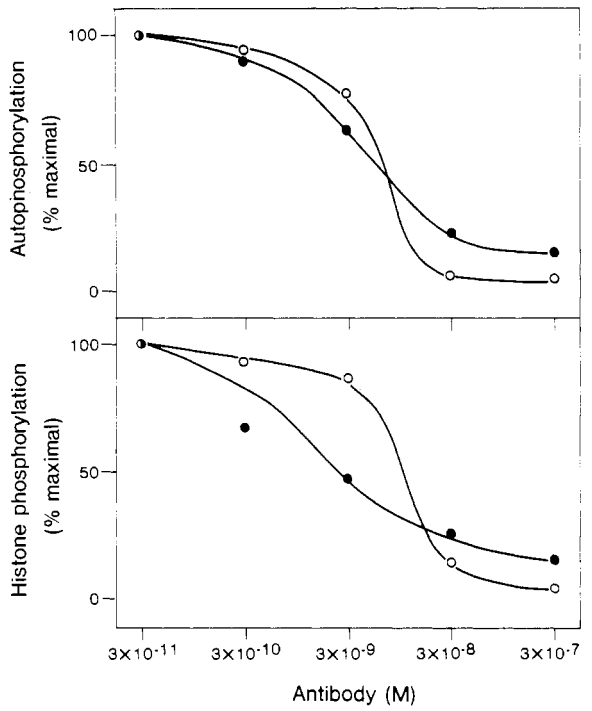

FIGURE 5: Effect of two β_2 antibodies on receptor kinase activity. Purified insulin receptor autophosphorylation (top) and histone H2b phosphorylation (bottom) were determined in the presence of the indicated concentrations of antibodies 17A3 (○) and 24B7 (●), as described under Methods. Similar results (±5%) were obtained in three separate experiments.

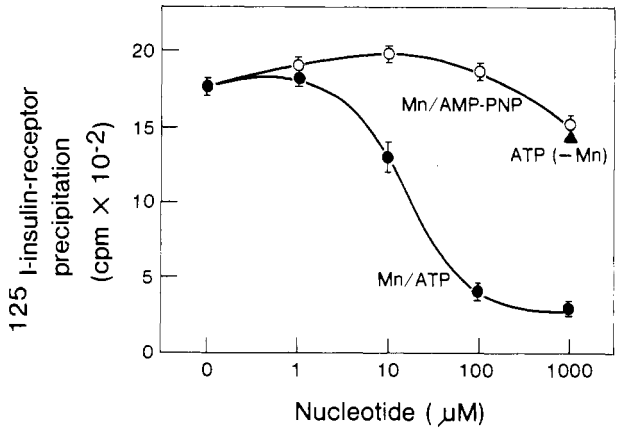

FIGURE 6: Effect of receptor phosphorylation on precipitation by β_2 antibody 20D9. Precipitation by 20D9-coated wells of receptor preincubated with indicated concentration of Mn/ATP (●), Mn/AMP-PNP (○), or ATP alone (▲) was performed as described under Methods. Values are means of triplicates ± SEM.

antibodies have been shown to purify the insulin receptor from various tissues (Morgan et al., 1985).

Of these 37 monoclonal antibodies, only 3 were found to bind to the extracellular domain of the receptor (Morgan et al., 1985). 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 hydrophylic than the rest of the receptor, since hydrophylic 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 hydrophylic (Ullrich et al., 1985; Ebina et al., 1985). 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 of 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 insulin receptors (Morgan et al., 1985).

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 yet 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 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 I, Figure 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 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 antigenic 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 of the receptor 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 4C), perhaps because it only recognizes certain glycosylated forms of the receptor. Proteolysis of the β subunit did not affect the binding of this antibody to the receptor (Morgan et al., 1985), 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 4A), their effect on the kinase activity of the receptor (Figure 3A), their affinity for autophosphorylated receptor (Figure 3B), and their ability to compete with other antibodies for receptor binding (Figure 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 2) and affinity and capacity for receptor (Table I), but they appear similar in their effect on the kinase (all moderate inhibitors, Figure 3A) and affinity for the autophosphorylated receptor (all slightly inhibited by autophosphorylation, Figure 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). Evidence for some heterogeneity of the β_2 class was found in the antigenic mapping studies (Figure 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 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 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 observation that the β_2 antibodies completely inhibited receptor autophosphorylation and did not recognize receptor after incubation with ATP and Mn^{2+} (Figure 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 for 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 protein product of the avian sarcoma virus UR2 (ros) (Ebina et al., 1985; 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.

Registry No. IGF-I, 67763-96-6; insulin, 9004-10-8; insulin receptor protein kinase, 88201-45-0.

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A Novel Catecholamine-Activated Adenosine Cyclic 3′,5′-Phosphate Independent Pathway for β-Adrenergic Receptor Phosphorylation in Wild-Type and Mutant S_{49} Lymphoma Cells: Mechanism of Homologous Desensitization of Adenylate Cyclase†

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ABSTRACT: Virtually all known biological actions stimulated by β-adrenergic and other adenylate cyclase coupled receptors are mediated by cAMP-dependent protein kinase. Nonetheless, "homologous" or β-adrenergic agonist-specific desensitization does not require cAMP. Since β-adrenergic receptor phosphorylation may be involved in desensitization, we studied agonist-promoted receptor phosphorylation during homologous desensitization in wild-type S_{49} lymphoma cells (WT) and two mutants defective in the cAMP-dependent pathway of β-agonist-stimulated protein phosphorylation (cyc^- cannot generate cAMP in response to β-adrenergic agonists; kin^- lacks cAMP-dependent kinase). All three cell types demonstrate rapid, β-adrenergic agonist-promoted, stoichiometric phosphorylation of the receptor which is clearly not cAMP mediated. The amino acid residue phosphorylated is solely serine. These data demonstrate, for the first time, that catecholamines can promote phosphorylation of a cellular protein (the β-adrenergic receptor) via a cAMP-independent pathway. Moreover, the ability of cells with mutations in the adenylate cyclase–cAMP-dependent protein kinase pathway to both homologously desensitize and phosphorylate the β-adrenergic receptors provides very strong support for the notion that receptor phosphorylation may indeed be central to the molecular mechanism of desensitization.

Hormones that elevate intracellular levels of cAMP induce their physiological effects through phosphorylation of specific target proteins mediated by cAMP-dependent protein kinase (Krebs & Beavo, 1979). Accordingly, key regulatory cellular proteins have been shown to become phosphorylated upon β-adrenergic agonist stimulation (Rubin & Rosen, 1985; Steinberg & Coffino, 1979; Steinberg & Agard, 1981; Nimmo & Cohen, 1977) and to be regulated in their functionality by such cAMP-mediated phosphorylation (Krebs & Beavo, 1979). It has also been shown that intracellular increases of cAMP achieved either through hormonal stimulation or by exposure of cells to membrane-permeable analogues of cAMP lead to identical protein phosphorylation (Steinberg & Coffino, 1979). All of these phosphorylation processes are catalyzed by cAMP-dependent protein kinases. In cells which are characterized by a transdominant regulatory mutation in the expression of the catalytic subunit of the cAMP-dependent protein kinase (i.e., the kin^- mutant of S_{49} lymphoma cells) (Steinberg et al., 1978) and which are therefore devoid of

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