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Insulin action is blocked by a monoclonal antibody that inhibits the insulin receptor kinase

(tyrosine kinase/oocytes/progesterone/microinjection)

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ABSTRACT **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.**

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 linked by disulfide bonds (1). 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 (2). Since many of the effects of insulin are caused by changes in the phosphorylation state of various proteins (3), 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*) there is reduced activity of the receptor kinase in cells from certain patients with diabetic syndromes characterized by lack of responsiveness to insulin (4, 5); (*ii*) phorbol diesters cause a parallel decrease in insulin responsiveness of cells and insulin-stimulated tyrosine kinase activity of the receptor (6); 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 (7–9). 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 (10, 11).

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 (12, 13), 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 (14, 15). Moreover, since these cells have a distinct receptor for progesterone (16) and progesterone also stimulates oocyte maturation (17), the effect of the antibodies on this response was also examined.

MATERIALS AND METHODS

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 (18) 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 crosslinking ^{125}I-labeled insulin (^{125}I-insulin) to purified placental receptor with disuccinimidyl suberate as described (19). In some experiments (see Fig. 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 crosslinking 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 (20) by a modification (21) of the procedure originally described by Kohler and Milstein (22). Supernatants from the resulting hybridomas were screened for anti-receptor antibodies with a plate immunoprecipitation assay as described (19). In brief, hybridoma supernatants were incubated in microtiter wells previously coated with anti-mouse IgG (40 μg/ml) (19). After 2 hr 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-hr 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.

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Cell Surface Labeling. IM-9 lymphoid cells (2×10^6 cells) were incubated 1 hr 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 were incubated with 250 ng of purified rat liver receptor. After 16 hr 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 (18). Twenty microliters of protein A-Sepharose coated with anti-mouse IgG was incubated 2 hr 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 hr at 4°C. After four washes, the precipitated proteins were subjected to $NaDodSO_4$/polyacrylamide 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 hr 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 μM 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 concentration, 10 μM) with or without 5 μg of histone H2b; after 1 hr 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 (23) 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 hr at 19°C, and healthy oocytes (20–25) then were transferred to Barth's medium (10 oocytes per 1 ml) containing 0.1% bovine serum albumin and the desired hormone. After 18 hr 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 (15) 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 1) and iodinated receptor (data not shown) in a plate precipitation assay (19). 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 1). This result suggested that the remaining 34 antibodies recognize an intracellular or transmembrane site on the receptor. Since previous work (24–26) 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.

Table 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

*Nonspecific labeling, determined by incubating cells with normal mouse immunoglobulin, 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 Fig. 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 (12).

The contribution of the β subunit to the sites recognized by the antibodies was further examined by utilizing the ability of crude collagenase preparations to extensively degrade the β subunit without affecting the α subunit (27). 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 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 (Fig. 1). In contrast, proteolysis had no effect on either the integrity of the α subunit or on the precipitation of the receptor with one of the antibodies to the extracellular domain (MC51) (Fig. 1). These

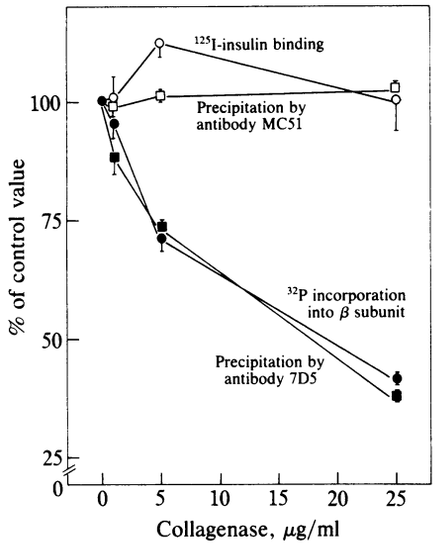

FIG. 1. Effect of β-subunit proteolysis on receptor function and antibody recognition. Each reaction mixture contained ≈250 ng of highly purified human placental insulin receptor in 5 μl (50 mM Hepes, pH 7.6/150 mM NaCl/0.1% Triton X-100). To monitor the integrity of the β subunit (●), receptor was autophosphorylated, collagenase-digested (27), and electrophoresed on reduced NaDodSO$_4$/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 hr at 24°C with 150,000 cpm of ^{125}I-insulin (120 Ci/g), treated with the crosslinking 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 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 hr 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 ± SEM of triplicates. The control values (100%) in the absence of collagenase were: 19,394 cpm (●); 1812 cpm (○); 1513 cpm (□); and 1157 cpm (■).

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 (12, 13), 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 1). In contrast, 33 of the 34 antibodies to the intracellular domain of the β subunit did precipitate the rat receptor (Table 1). Additional studies with 1 of these antibodies (7D5) indicated that this antibody could precipitate the human, mouse, chicken, and frog receptors with almost equal affinity (Fig. 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 (Fig. 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% (Fig. 3). The effects

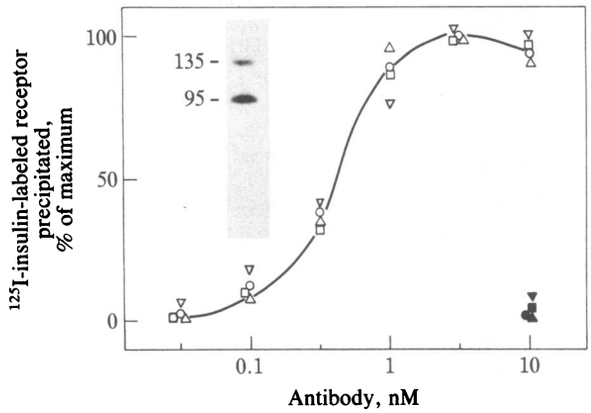

FIG. 2. Precipitation of mammalian, avian, and amphibian insulin receptors by antibody 7D5. Microtiter wells were coated with normal mouse immunoglobulin (closed symbols) or various concentrations of antibody 7D5 (open symbols) as described. In one series of wells, 50 μl of ^{125}I-insulin-labeled human (8100 cpm) (○), mouse (1600 cpm) (□), and chicken (8100 cpm) (△) receptor was added to antibody-coated wells, incubated for 16 hr at 4°C, washed, and assayed for radioactivity. In another series, purified frog oocyte receptor (▽) was added to antibody-coated wells and incubated 16 hr 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 sizes indicated in kDa.

of these antibodies on the kinase activity of the frog oocyte receptor kinase paralleled that found with the human receptor, although some small differences were noted (Fig. 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 (Fig. 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 (Fig. 4). In contrast, another antibody (1G2) at 300 nM

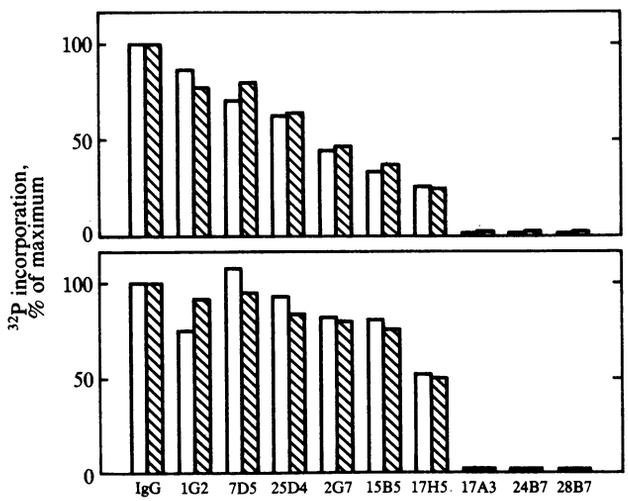

FIG. 3. Effect of nine monoclonal antibodies on the kinase activities of purified human placental and frog oocyte insulin receptors. Purified human placental (*Upper*) or *Xenopus* oocyte (*Lower*) insulin receptors were incubated with 100 nM of the indicated antibodies and tested for their ability to either autophosphorylate (open bars) or phosphorylate histone (hatched bars) as described. All values are means of duplicates.

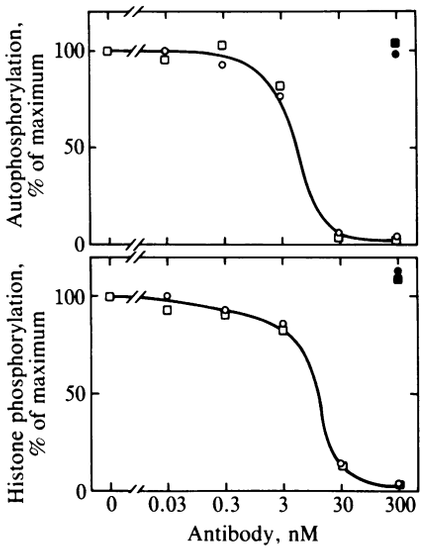

FIG. 4. Comparison of the inhibitory potency of antibody 17A3 on the kinase activities of human and frog insulin receptors. Purified human placental (○) or *Xenopus* oocyte (□) insulin receptors were incubated with antibody 17A3 (open symbols) or antibody 1G2 (closed symbols). Kinase activity was then 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.

had no effect on either autophosphorylation or phosphorylation of histone by either receptor (Fig. 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 hr. In contrast, in the presence of 1 μM 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), an inhibition of the insulin response by 80%. In two additional experiments, the inhibition observed with 13 nM 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). 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).

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 hr all bound the same amount of ^{125}I-insulin (data not shown).

Since progesterone also can stimulate maturation of oocytes (17), 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). The lack of effect of the inhibiting antibody 17A3 on the progesterone response was demonstrated in two additional experiments.

Table 2. Effect of microinjection of anti-receptor antibodies on oocyte maturation

Microinjection		Positive oocytes after hormonal treatment, %			
			Insulin		Progesterone
Antibody	nM	None	10 μM	1 μM	(1 μM)
None		0	57	62	96
Mouse IgG	13	10	45	57	74
Noninhibiting anti-receptor IgG (1G2)	13	0	68	44	80
Inhibiting 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 concentration 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 μl per oocyte. NT, not tested.

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 1). Also, proteolysis of the β subunit inhibits the ability of these same 34 antibodies to bind to the receptor (Table 1 and Fig. 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 (Fig. 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 1). Furthermore, 1 of these antibodies (7D5) precipitated the human, mouse, chicken, and frog receptors with almost equal high affinity (Fig. 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 (Fig. 4). In contrast, none of the antibodies to the extracellular domain recognized the rat liver insulin receptor (Table 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 (28).

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 (Fig. 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* (2, 29). 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 (24, 25).

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). 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 (16). 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 and Fig. 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 (Fig. 4), had no effect on the ability of the oocytes to respond to insulin (Table 2).

The importance of tyrosine kinases in initiating maturation of oocytes has been suggested by the finding that microinjection of the src tyrosine kinase into oocytes accelerates the rate of progesterone-induced maturation (30). 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 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.

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