Insulin-Ricin B Hybrid Molecules Mediate an Insulin-associated Effect on Cells Which Do Not Bind Insulin*

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A hybrid molecule was constructed by covalently linking, by a disulfide bridge, the hormone insulin to the binding subunit B of the plant toxin ricin (specificity: Gal, GalNAc). Monolayer-cultured MDCK cells, which lack detectable levels of specific plasma membrane 125I-insulin binding but which readily bind 125I-insulin-ricin B, were used in these studies. Binding of insulin-ricin B to these cells could be displaced by lactose and ricin B, but not by insulin. The biological activity of the hybrid, as measured by [14C]glucose incorporation into glycogen, was stimulated in a dose-dependent manner by the hybrid (10^-11 - 10^-8 M), whereas glycogen production was not stimulated by insulin alone. The stimulated glycogen labeling in response to the hybrid was also inhibited by lactose and ricin B. When ricin B alone was tested over the same range of concentrations, stimulation of glycogen synthesis was not observed, nor was there any evidence for stimulation when insulin and ricin B were added simultaneously. These data suggested that alternate cell surface receptors (i.e. ricin B receptors) may substitute for specific receptors (i.e. insulin receptors) to convey intracellular metabolic control signals in this cell line.

While the binding of insulin to its cell surface receptor initiates a chain of biochemical events leading to characteristic intracellular effects of the hormone, the molecular mechanisms underlying these events remain unknown (1). Two general mechanisms for the action of insulin have been proposed. One suggested mechanism is that insulin binding to its receptor results in the activation of a membrane-associated enzyme or transport system which may in turn generate an intracellular messenger mediating hormone action, analogous to the production of cAMP as the second messenger for other peptide hormones (2, 3). Alternatively, it has been suggested that receptor-bound insulin may enter the cell and act as its own second messenger to convey appropriate biological action signals (4, 5). Such effects could result from a direct intracellular action of the intact insulin molecule or through less direct effects promoted by production of biologically active fragments of the hormone (5). Since insulin is known to mediate a broad range of cellular responses from acute metabolic effects to chronic growth effects, it also remains possible that both surface and internalization-mediated action mechanisms may be operative in differentially controlling these diverse hormonal effects.

To probe possible cellular mechanisms for insulin action, hybrid molecules possessing properties of both hormone and toxin molecules were synthesized. The plant toxin ricin is known to specifically bind by its B chain to galactose or N-acetylglactosamine residues of cell surface macromolecules. Bound ricin molecules are reportedly internalized via adsorptive endocytosis ultimately allowing the toxic A chains to enter the cytoplasm and inhibit protein synthesis at ribosomal sites (6-8). For the present studies, the ricin B chain was covalently coupled to insulin as first reported by Roth et al. (9) to produce hybrid molecules which are capable of binding and entering cells via the ricin receptor, while at the same time maintaining their ability to promote intracellular responses characteristic of insulin (Fig. 1, A and B). The biological efficacy of such hybrid molecules was then tested on monolayer cultures of MDCK cell lines, a line recently shown to have nearly undetectable levels of plasma membrane insulin-binding sites. Results of the presently reported studies demonstrated that presentation of insulin-ricin B hybrid molecules to MDCK cells produced the insulin-associated effect of stimulated glycogen synthesis. Such experimental results suggested that plasma membrane receptors for ricin in this cell line were effective functional alternates for insulin receptors as initial sites for hormone interaction (Fig. 1C).

EXPERIMENTAL PROCEDURES

Materials

Monocomponent porcine insulin was generously supplied by Dr. William Bromer, Lilly Research Laboratories, Indianapolis, IN. [U-13C]glucose, Na+[^1]I, [125I]-protein A, NCS tissue solubilizer, and OCS counting fluid were purchased from Amerham Corp. Culturing supplies including Swim's-77 medium, penicillin-streptomycin, and trypsin were purchased from Gibco Laboratories, Grand Island, NY. Horse serum and fetal calf serum were obtained from KC Biologicals, Lenexa, KA. PD-10 columns and the molecular coupling reagent N-succinimidyl-3-(2-pyridyldithio)propionate were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Rabbit serum containing antibodies to the insulin receptor (A410) was generously provided by Dr. Steven Jacobs (Wellcome Research Laboratory, Research Triangle Park, NC).

Methods

Cell Culture—The continuously cultured MDCK cell line, originally derived from normal dog kidney (10), was obtained for use in

1 The abbreviations used are: MDCK, Madin-Darby canine kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H4, H4-II-EC3 hepatoma cells.
2 M. Fehmann, unpublished results.
these studies from the American Type Culture Collection, Rockville, MD. The insulin-binding, minimal-deviation H4 hepatoma cell line also was previously described (11) and was originally provided by Dr. Van Potter, McArdle Research Laboratories, Madison, WI. Cells were routinely cultured in Swinn’s medium supplemented with 2% of additional glucose, 12 mg of cysteine, 20 mg of phenol red, 2.2 g of NaHCO3, 0.77 g of glutamine, and 0.2 g of CaCl2 per liter and also containing 10% horse serum and 2.5% fetal calf serum. Cells were grown in a humidified incubator gassed with 5% CO2 in air at 37°C.

Ricin B Preparation—Ricin extracted from castor beans was purified by affinity chromatography on Sepharose-4B and ion exchange chromatography as previously described (12). Following treatment of ricin with β-mercaptoethanol the binding chains were separated from the N-acytelyglactosamine residues by ion exchange chromatography (12). Protein concentration was estimated by the technique of Waddell (14).

Synthesis of Insulin-Ricin Hybrid Molecules—The synthesis and purification of the insulin-ricin B hybrid was based on the methods of Shechter et al. (15) and Carlsson et al. (16). The synthesis is outlined in Fig. 2, and a detailed description of the methods has been published by Roth et al. (9). To assure retention of the biological activity of insulin, coupling to the hormone was limited to linkage through the α-amino group of the lysine residue in position 29 on the insulin B chain. Retention of function of the insulin portion of the hybrid molecule was judged by evaluating the ability of the hybrid to displace labeled insulin binding to H4 hepatoma cells as compared to displacement by native insulin standards (11). For this evaluation, the binding was examined in the presence of 10 mM lactose to prevent interaction with any ricin receptor sites. Assuming a 1:1 insulin:ricin coupling ratio, these binding assays were used to express the concentration of the hybrid preparation in terms of insulin bioequivalent activity.

Iodination of Insulin and Hybrid Molecules—Insulin was iodinated using a chloramine-T technique previously described (17), and insulin-ricin B hybrid molecules were iodinated in the same way. However, 125I-labeled hybrid was separated from free Na125I using Pharmacia PD-10 columns equilibrated with phosphate-buffered saline containing 30% glycerol.

125I-Insulin-Ricin B and 125I-Insulin Binding Assays—Confluent monolayer cultures of MDCK or H4 cells (1–3 X 106 cells/60-mm plate) were washed with phosphate-buffered saline and incubated 10 min at 30°C with 1.4 ml of Swinn’s medium at pH 7.4 containing 20 mM Hepes, 5 mM sodium bicarbonate, and 1% bovine serum albumin, with various concentrations of lactose, purified ricin B chain preparations, or native insulin added as specified. 125I-Insulin-ricin B (0.1 ml) or 125I-insulin (0.1 ml) was then added to final concentrations of ~1010 M (200,000 cpm). After the indicated time of incubation at 30°C, the medium was aspirated and monolayers were washed with ice-cold phosphate-buffered saline, solubilized with 2 ml of 88% formic acid, and counted to determine bound radioactivity. Total 125I-insulin-ricin B binding and 125I-insulin binding to cells was corrected for nonspecific, cell-associated radioactivity by subtracting counts bound in the presence of 10 mM lactose or 20 μg/ml of native insulin respectively.

Incorporation of D-[U-14C]Glucose into Glycogen—The assay for insulin or insulin-ricin B-stimulated incorporation of [14C]glucose into glycogen monolayer cultures of MDCK cells showed rapid and specific binding of 125I-insulin-ricin B hybrid, with maximum binding approaching 6% of the total radioactivity added. However, these cell cultures were essentially devoid of 125I-insulin binding sites when total cell-associated 125I-insulin was corrected for nonspecific binding (0.25–0.4% of total radioactivity added) (Fig. 3). In contrast, control cultures of insulin-sensitive H4 hepatoma cells (11) showed specific binding of both 125I-insulin-ricin B hybrid (60-min binding = 4.3% of total radioactivity added) and 125I-insulin (60-min binding = 1.0% of total radioactivity added) (Fig. 3).

Immunological detection of insulin receptor components was accomplished by incubation of monolayer-cultured cells with rabbit serum containing antibodies to purified insulin receptors. Following rinsing, cell-bound antibody was detected by a secondary incubation with 125I-labeled protein A. The amount of cell-associated radioactivity on antibody-treated H4 cells was markedly increased over background binding.

**RESULTS**

Monolayer cultures of MDCK cells showed rapid and specific binding of 125I-insulin-ricin B hybrid, with maximum binding approaching 6% of the total radioactivity added. However, these cell cultures were essentially devoid of 125I-insulin binding sites when total cell-associated 125I-insulin was corrected for nonspecific binding (0.25–0.4% of total radioactivity added) (Fig. 3). In contrast, control cultures of insulin-sensitive H4 hepatoma cells (11) showed specific binding of both 125I-insulin-ricin B hybrid (60-min binding = 4.3% of total radioactivity added) and 125I-insulin (60-min binding = 1.0% of total radioactivity added) (Fig. 3).

**Fig. 1.** Cell surface binding of insulin, ricin B, and insulin-ricin B hybrid molecules. A, the initial step in the interaction of insulin with target cells is the binding of the hormone to its specific cell surface receptor. Similarly, ricin B chain is capable of binding to cell surface receptors. B, when insulin is coupled covalently to the ricin B chain, the resulting hybrid molecule is potentially capable of binding at the cell surface through either insulin-specified receptors or ricin-specified carbohydrate residues. C, since MDCK cells appear to lack functional insulin binding sites, the hybrid insulin-ricin B molecules presumably bind via ricin B receptor sites.

**Fig. 2.** Scheme for synthesis of insulin-ricin B hybrid molecules. Blocking, NH2-terminal amino groups of insulin were reversibly blocked by citraconylation. Derivatization, insulin was activated by reaction with the heterobifunctional cross-linking reagent, N-succinimidyl-3-(2-pyridyldithio)propionate. Deblocking, "protective groups were removed from α-amino groups of insulin. Coupling, derivatized insulin was reacted with the free sulfhydryl of purified ricin B chain.

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levels and was dependent upon the concentration of antibody-containing serum included in the initial incubation medium (Table I), thus immunologically confirming the presence of insulin receptors in H4 cells. In contrast, binding of 125I-protein A to MDCK cells previously treated with anti-insulin receptor antibodies failed to increase above background binding levels (Table I), indicating a lack of immunologically recognizable insulin receptor components on this cell line.

Promotion of glycogen synthesis is a characteristic action of the hormone insulin. The effect of insulin is largely due to conversion of the enzyme glycogen synthase from an inactive D form to an active I form (18). As it has been shown that incorporation of glucose into glycogen directly reflects synthase activity (19, 20), we have selected and assayed for this action as a simpler correlate. When MDCK cell monolayer cultures were incubated with various concentrations of insulin (1.67 × 10^-10-1.67 × 10^-8 M) and 3 μCi of [14C]glucose, production of 14C-labeled glycogen was not stimulated above basal levels (Fig. 4). Incubation of the cells with hybrid insulin-racin B preparations at comparable concentrations, however, resulted in 14C-glycogen production maximally stimulated more than 50% over basal levels (Fig. 4).

MDCK monolayer cultures were incubated with insulin-racin B hybrid (1.67 × 10^-10 M) with tracer 125I-hybrid and with further addition of lactose at indicated concentrations. Under these experimental conditions, >90% of the cell-associated 125I-insulin-racin B was displaced by inclusion of 10 mM lactose in the incubation medium (Fig. 5A). Insulin (20 μg/ml) was ineffective in displacing 125I-insulin-racin B binding from these cells (Table II). In additional control experiments, lactose at comparable concentrations had no effect on the binding of 125I-insulin to H4 hepatoma cell cultures. Altogether, these data suggested that hybrid binding to the MDCK cells occurred specifically and exclusively via ricin B receptor sites.

Lactose effects at these concentrations shown to actively displace hybrid binding (0.3-10 mM) were further examined in parallel experiments evaluating insulin-racin B hybrid-stimulated glycogen production. Such experiments demonstrated that hybrid-stimulated incorporation of [14C]glucose into glycogen was also inhibited in a dose-dependent manner.

![Graph](http://www.jbc.org/)

**Fig. 3. Time course of specific 125I-insulin and 125I-hybrid binding to MDCK and H4 cells.** Confluent monolayer cultures of MDCK cells (2.9 × 10^6 cells/plate) and H4 (2.8 × 10^6 cells/plate) were incubated 60 min at 30 °C with 125I-insulin (200,000 cpm) or 125I-insulin-racin B (200,000 cpm) in a total of 1.5 ml of binding medium. Monolayers were subsequently rinsed, solubilized, and counted for cell-associated radioactivity. Data were corrected for nonspecific binding as described under "Methods." MDCK cells with insulin (●-●), with hybrid (○-○); H4 cells with insulin (●-●), with hybrid (○-○). Data points represent the mean value ± S.D. for triplicate determinations.

**Table I**

<table>
<thead>
<tr>
<th>Treatment reagent</th>
<th>Dilution in medium</th>
<th>125I-Protein A bound*</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>1:200</td>
<td>1,661 ± 117</td>
<td>1,433 ± 12</td>
</tr>
<tr>
<td>Anti-insulin receptor serum</td>
<td>1:200</td>
<td>19,436 ± 1,594</td>
<td>1,292 ± 114</td>
</tr>
<tr>
<td>Normal serum</td>
<td>1:2,000</td>
<td>1,753 ± 167</td>
<td>1,835 ± 54</td>
</tr>
<tr>
<td>Anti-insulin receptor serum</td>
<td>1:2,000</td>
<td>10,214 ± 281</td>
<td>1,937 ± 189</td>
</tr>
</tbody>
</table>

*Mean of triplicate values ± S.D.

**Fig. 4. Effect of hybrid or insulin on 14C-glycogen production in MDCK cells.** MDCK monolayer cultures (4 × 10^6 cells/60-mm plate) were incubated for 2 h at 37 °C in medium containing 3 μCi of [14C]glucose and the indicated concentrations of hybrid (●-●) or insulin (○-○). The cellular glycogen was then extracted and the radioactivity determined by liquid scintillation counting. Data points represent the mean value ± half range for duplicate determinations.
FIG. 5. Effect of lactose on hybrid binding to MDCK cells (A) and on basal and hybrid-stimulated 
\( ^{14} \text{C}-\text{glycogen production} \) (B). A, MDCK monolayer cultures were incubated with insulin-ricin \( B \) hybrid (1.67 \( \times \) 10\(^{-9} \) M) along with tracer \( ^{125} \text{I}-\)hybrid and indicated concentrations of lactose for 45 min at 37 \( ^{\circ} \)C. Plates were then rinsed and cells were solubilized for counting. Data points represent the mean value \( \pm \) S.D. for triplicate determinations. B, in experiments parallel to those shown in A, MDCK monolayer cultures were incubated for 45 min at 37 \( ^{\circ} \)C in the absence (○—○) or presence (●—●) of insulin-ricin \( B \) hybrid (1.67 \( \times \) 10\(^{-9} \) M) with 3 \( \mu \)Ci of \( ^{14} \text{C}-\)glucose and lactose at indicated concentrations. Following incubation, cellular glycogen was extracted and radioactivity was determined. Data points represent the mean value \( \pm \) S.D. for triplicate determinations.

| Table II |
|-----------------|-----------|
| Effect of insulin and lactose on \( ^{125} \text{I}-\)hybrid binding to MDCK cells |
| \(^{125} \text{I}-\)Hybrid bound | % control |
| Control medium | 23,674 ± 2,794 | 100 |
| Medium with 10 mM lactose | 844 ± 71 | 4 |
| Medium with 20 \( \mu \)g/ml of insulin | 23,576 ± 303 | 100 |
| Medium with lactose and insulin | 829 ± 109 | 4 |

Mean of triplicate values \( \pm \) S.D.

by the presence of lactose (Fig. 5B). These results indicated that the hybrid stimulation of the insulin-associated response, like the hybrid binding, was mediated through an interaction with the ricin receptor. Control data further demonstrated that lactose had no effect on basal incorporation of \( ^{14} \text{C} \) glucose into glycogen (Fig. 5B).

In subsequent experiments, MDCK monolayer cultures were incubated with insulin-ricin \( B \) hybrid (\( \sim 1.67 \times 10^{-9} \) M), with tracer \( ^{125} \text{I}-\)hybrid added, and with further addition of purified ricin \( B \) chain preparation at indicated concentrations. At 10\(^{-7} \) M, the highest concentration tested, unlabeled ricin \( B \) inhibited \( \sim 50\% \) of the total \( ^{125} \text{I}-\)insulin-ricin-\( B \) binding (Fig. 6A). Comparable experiments testing the effects of ricin \( B \) on basal and hybrid-stimulated \( ^{14} \text{C}-\)glycogen production were also performed. The results indicated that the hybrid-stimulated biological response, as the hybrid binding, was partially inhibited by ricin \( B \) preparations at the concentrations tested. Ricin \( B \) chain alone was nonstimulating within the indicated concentration range (Fig. 6B). Mixtures of purified ricin \( B \) chain and native insulin were tested for possible synergistic effects in promoting \( ^{14} \text{C}-\)glycogen production from \( ^{14} \text{C} \) glucose. At all concentrations tested, no evidence of such effects was obtained (data not shown). Furthermore, the ricin \( B \) preparation (10\(^{-7} \)–10\(^{-9} \) M) failed to augment specific binding of \( ^{125} \text{I} \) insulin to the cells (Table III).

FIG. 6. Effect of ricin \( B \) on hybrid binding to MDCK cells (A) and on basal and hybrid-stimulated \( ^{14} \text{C}-\)glycogen production (B). A, MDCK monolayer cultures were incubated for 45 min at 37 \( ^{\circ} \)C with insulin-ricin \( B \) hybrid (1.67 \( \times \) 10\(^{-9} \) M) along with tracer \( ^{125} \text{I}-\)hybrid and indicated concentrations of purified ricin \( B \) preparations. Plates were then rinsed and cells solubilized for counting. Data points represent the mean value \( \pm \) S.D. for triplicate determinations. B, in experiments parallel to those shown in A, MDCK monolayer cultures were incubated for 45 min at 37 \( ^{\circ} \)C in the absence (○—○) or presence (●—●) of insulin-ricin \( B \) hybrid (1.67 \( \times \) 10\(^{-9} \) M) with 3 \( \mu \)Ci of \( ^{14} \text{C}-\)glucose and lactose at indicated concentrations. Cellular glycogen was extracted and radioactivity was determined. Data points represent the mean value \( \pm \) S.D. for triplicate determinations.
TABLE III

Effect of ricin B on specific binding of \textsuperscript{125}I-insulin to MDCK cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Specific</th>
<th>CPM</th>
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<tbody>
<tr>
<td>Control medium</td>
<td>857 ± 64</td>
<td>159</td>
</tr>
<tr>
<td>Medium with ricin B, \textsuperscript{10}^{-7} M</td>
<td>735 ± 49</td>
<td>37</td>
</tr>
<tr>
<td>Medium with ricin B, \textsuperscript{10}^{-8} M</td>
<td>727 ± 63</td>
<td>29</td>
</tr>
<tr>
<td>Medium with ricin B, \textsuperscript{10}^{-9} M</td>
<td>749 ± 119</td>
<td>51</td>
</tr>
<tr>
<td>Medium with insulin, 2 × 10^{-6} M</td>
<td>698 ± 86</td>
<td></td>
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*Corrected for nonspecific binding as described “Methods.” Mean of triplicate values ± S.D.

DISCUSSION

To investigate the cellular mechanism of insulin action, we have utilized hybrid molecules which possess properties of both hormone and toxin molecules. Ricin is composed of two chains: the 34,000-dalton \( B \) chain is responsible for carbohydrate-specific (Gal, GalNAc) cell surface binding leading to uptake of the 32,000-dalton toxic \( A \) chain which inhibits protein synthesis (6–8). For the present studies, we have covalently coupled the \( B \) chain subunit of ricin to insulin to produce a hybrid molecule which maintains hormonal potential, but with additional binding capabilities. Such hybrid molecules with altered binding properties have been produced previously by coupling various plant and animal toxin components to human chorionic gonadotropin (21, 22), low density lipoprotein (20), insulin (4, 9, 24, 25), monophosphopentamannose (23), and lectins (26, 27).

Roth et al. (9) demonstrated insulin-like activities (stimulation of \([\text{H}]\)aminoisobutyrate uptake, tyrosine aminotransferase induction) of insulin-ricin \( B \) hybrids on HTC hepatoma cells. These cells reportedly possessed a small number of insulin receptors, but appeared to bind the hybrid primarily to ricin \( B \) receptors rather than insulin receptors. Although the insulin receptor number was low in the HTC cells, it remained possible that the hybrid conjugate could have mediated insulin actions by affecting the interaction of insulin with its own receptors.

Our experiments have confirmed and extended the studies of Roth et al. (9) by showing that presentation of insulin-ricin \( B \) hybrid molecules to MDCK cells, which apparently lack insulin receptors, resulted in stimulated glycogen production. In these cells, insulin alone had no effect on glycogen synthesis. Plasma membrane receptors for ricin in this cell line thus appeared to act as functional alternates for insulin receptors as initial sites for hormone interaction.

In accordance with the role of the \( B \) subunit of native ricin in mediating binding, endocytotic uptake, and cytosolic delivery of the toxic \( A \) subunit (6–8), it is plausible that the ricin \( B \) portion of the hybrid molecule may similarly mediate translocation of the hormone insulin, or some fragment thereof, to appropriate intracellular site(s) of action. There is abundant morphological and biochemical evidence establishing the internalization of receptor-bound insulin (17, 28–34), although there is at the present time only limited evidence supporting the possibility of direct regulation of metabolic activities by this internalized hormone (35). Furthermore, reports that antibodies against the insulin receptor as well as the lectin concanavalin A mimic many of insulin's actions (36) would not appear to support the concept of a direct intracellular action by the hormone itself.

Insulin has also recently been reported to promote glycogen synthesis through an intracellular mediator (37) which appeared to be generated by a proteolytic event occurring at the plasma membrane (38). It was hypothesized that this mediator may enter cells through membrane pores somehow formed by the hormone-receptor interaction (38). Parallel to its function in the native ricin molecule, the ricin \( B \) portion of the hybrid molecule could conceivably be involved in such pore formation through an interaction with its own membrane glycoprotein receptor. Since fidelity of the mediator with the insulin receptor is not implicit, it is possible that such a mediator might be produced even in the absence of the insulin receptor. In fact, provisional estimates have suggested that mediator is present at levels that are 100–1000 times greater than normal insulin receptor concentrations (38). In our experiments, ricin \( B \) chain alone was unable to promote glycogen production, so an action of the insulin portion of the hybrid molecule would appear to be a requisite for production of the putative mediator in this mechanism. As it has been shown that cross-linking of insulin receptors is essential for mediating biological responses to the hormone (2), the insulin portion of the hybrid molecule may indirectly accomplish such receptor linking. The precise action of the insulin portion of the hybrid in mediating biological response, however, presently remains undefined.

Further possible explanations for the observation of stimulated glycogen production by hybrid insulin-ricin \( B \) in the insulin nonbinding MDCK cells may lie in the nature of the insulin receptor defect in this cell line. It is possible that interaction of hybrid molecules with cell surface glycoproteins might sufficiently alter the membrane to allow exposure to previously cryptic insulin receptor sites. We were unable to generate any experimental evidence for such an action, however, since ricin \( B \) did not increase binding of \textsuperscript{125}I-insulin to the cells. It is also possible that insulin receptors may be present in MDCK cells, but unable to bind insulin, a defect overcome by the surrogate binding capabilities of the ricin \( B \) moiety of the hybrid. Such interaction might presumably restore normal function to the defective receptors, thus leading to appropriate cellular responses. We were not able, however, to generate any experimental evidence indicating such a receptor defect in the MDCK cells.

In summary, we have reported that MDCK cells appear to lack specific cell surface insulin receptors since we were unable to experimentally demonstrate either \textsuperscript{125}I-insulin binding to monolayer cultures or biological responsiveness to insulin. We were also unable to detect insulin receptor components in MDCK cells using immunological labeling techniques. We have, thus, interpreted our studies indicating control of glycogen metabolism in these cells by insulin-ricin \( B \) hybrid molecules as evidence that an insulin action can be evoked via an initial cellular interaction with receptors other than the insulin receptor, i.e. ricin receptors (Fig. 1). We have demonstrated that the ricin \( B \) portion of the hybrid molecule was responsible for binding to cell surface receptors and may additionally be responsible for the internalization of insulin or some molecular second messenger to mediate this intracellular response. Since ricin \( B \) chain alone did not promote glycogen production, the insulin component of the hybrid molecule appeared to be essential for generating the cellular response, and we have proposed several possible explanations for this observation. Further studies using toxin-hormone hybrid molecules as described in the present report should help to discriminate among these possible mechanisms for insulin-mediated control of intracellular responses or to facilitate proposals of new alternative explanations. Since insulin mediates numerous cellular responses ranging from acute metabolic effects to chronic growth effects, it will also be important for a better understanding of the underlying action mechanism to determine how many of these actions can be evoked by the hybrid molecules described in the present study.
Acknowledgments—We thank Dr. Max Fehlman for suggesting use of the MDCK cell line. We also thank Eric Morton and Joanna Moore for technical assistance in performing these experiments, and Cynthia Holmes and Carolyn Barnick for providing editorial and typing expertise in the preparation of this manuscript.

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Insulin-ricin B hybrid molecules mediate an insulin-associated effect on cells which do not bind insulin.

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