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INSULIN RECEPTORS ON CHINESE HAMSTER OVARY (CHO) CELLS: ALTERED INSULIN BINDING TO GLYCOSYLATION MUTANTS

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SUMMARY: We have identified and characterized insulin receptors on Chinese hamster ovary (CHO) cells. Insulin binds in a time, temperature and pH dependent fashion and insulin analogues compete for ^{125}I-insulin binding in order of their biological potencies. Furthermore, two CHO cell glycosylation mutants, B4-2-1, lacking high mannose containing glycoproteins, and Lec 1.3c, lacking complex carbohydrate containing glycoproteins, bind insulin with a much higher and lower affinity respectively than wild type cells. This is the first report of insulin receptors on CHO cells and the first to use glycosylation mutants to study the effects of abnormal carbohydrates on insulin binding.

In the plasma membrane, the insulin receptor is composed of two major disulfide linked glycoprotein subunits (1,2). Previous studies correlating glycosylation of the receptor with insulin binding have used enzymatic digestion of cells or membranes (3,4,) or inhibitors of glycosylation (5) to approach the question of the role of carbohydrates in insulin binding and action. We have studied the binding of ^{125}I-insulin to wild type (WTB) CHO cells, first, to demonstrate that CHO cells bind insulin and, second, to establish the proper conditions for the binding assay. These steps are required prior to using CHO cell lines with intrinsic genetic defects in glycoprotein processing to evaluate the role of carbohydrates in the binding of insulin to its receptor.

Two Chinese hamster ovary cell mutants, designated B4-2-1 and Lec 1.3c were screened in the current studies. B4-2-1 cells (6) are unable to synthesize mannosylphosphoryldolichol (7) which is the normal donor of the final 4-5 mannose residues to the dolichol phosphate mannose intermediate

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(8). These cells, therefore, transfer shortened high mannose structures to newly synthesized proteins. Once transferred, the truncated oligosaccharide may be processed normally or, as happens more frequently, less completely than normally. Lec 1.3c cells lack the enzyme N-acetylglucosamine transferase I and therefore are not able to process high mannose type oligosaccharides to complex forms (9).

These two mutant cell lines were used to establish the feasibility of utilizing CHO cell glycosylation mutants to delineate the role of carbohydrates in receptor function in an unperturbed cell culture system.

Materials and Methods

Cell Culture:

CHO cells were grown at 34°C in a humidified CO_2 atmosphere in Eagle's minimal essential medium (Biofluids, Rockville, MD) supplemented with non-essential amino acids (GIBCO, Grand Island, N.Y.) and 5% fetal bovine serum (Sterile Systems, Logan, Utah) and subcultured at a 1:15 split ratio weekly. For binding studies, cells were removed from $75cm^2$ flasks (Falcon) with 0.1% trypsin in calcium and magnesium free PBS and plated in 6-well cluster plates (Linbro) at a density of approximately $2\text{-}5x10^5$ cells per well.

Binding assay:

When the cells approached confluence, usually in 3-4 days, the growth medium was removed, the cells washed once with PBS, and 1.2mls binding buffer (100mM Hepes, 5mM KCl, 1.2mM $MgSO_4$, 118mM NaCl, 8.8mM D-glucose, 1% bovine serum albumin, pH8.) added at 15°C.

^{125}I-Insulin (New England Nuclear, Boston, MA: 370μCi/μg) and appropriate concentrations of porcine insulin (Elanco Products, Indianapolis, Indiana) were then added to bring the final volume per well to 1.5mls. Incubation was continued at 15°C for 4 hrs. The cells were then quickly washed 3 times with ice cold PBS and solubilized with 1ml 0.1% sodium dodecyl sulfate. Aliquots were removed for protein determination by the method of Lowry et al (10) and the rest counted in a Searle autogamma counter.

Results

^{125}I-Insulin bound to WTB cells at all temperatures tested: 15°C, 22°C and 34°C. As expected, binding was highest at 15°C, achieving a plateau between 3 and 6 hours of incubation (data not shown). At 22°C 50% as much ^{125}I-insulin was bound per mg cell protein and this was reduced to only 30% as much with a 34°C incubation. An incubation of 4 hours at 15°C was used for all subsequent experiments.

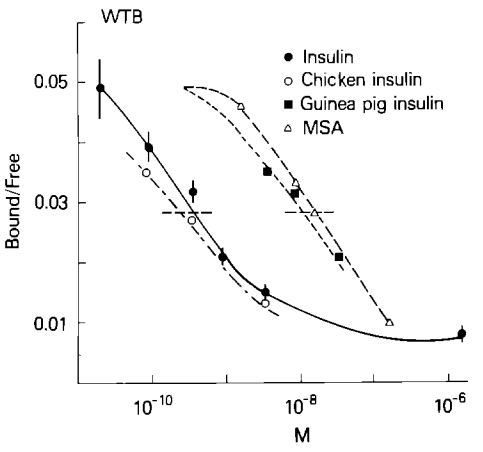

Figure 1. ^{125}I-insulin binding to wild type CHO cells. Cells were prepared and processed as described in Methods. Bound/Free ratio normalized to mg cell protein is plotted as a function of the concentration of competitor added: porcine insulin (mean±SEM), chicken insulin (Eli Lilly, Indianapolis, Indiana), guinea pig insulin (Novo Industries, The Netherlands), Multiplication Stimulating Activity (Collaborative Research, Waltham, MA) Horizontal dashed lines indicate 1/2 Bmax.

The pH optimum for binding to WTB cells was 8 with a 40% decrease at both pH 7.4 and pH 9 (data not shown). Competition curves using porcine insulin gave a half maximal displacement of 3-4x10^{-10}M (Fig. 1, Table I). Insulin analogues were tested and competed for ^{125}I-insulin binding as predicted from their bioactivities. Chicken insulin was twice as potent as porcine insulin, guinea pig insulin 4% as potent and the insulin-like growth factor MSA was 1-2% as effective as insulin in competing for labelled insulin (Fig. 1). ^{125}I-Insulin could be dissociated from its receptor after steady state binding had been achieved by the addition of

Table 1
Insulin Binding to CHO Cells

Cell line	Number of Exps.	B/F	p value*	1/2 Bmax (ng/ml)	p value*
WTB	9	0.047 ± 0.003**		2.15 ± 0.8	
B4-2-1	8	0.178 ± 0.015	≪.0005	0.88 ± 0.5	≪.001
Lec1.3c	6	0.021 ± 0.003	≪.0035	8.50 ± 4.0	≪.0005

*compared to WTB
**mean ±SEM

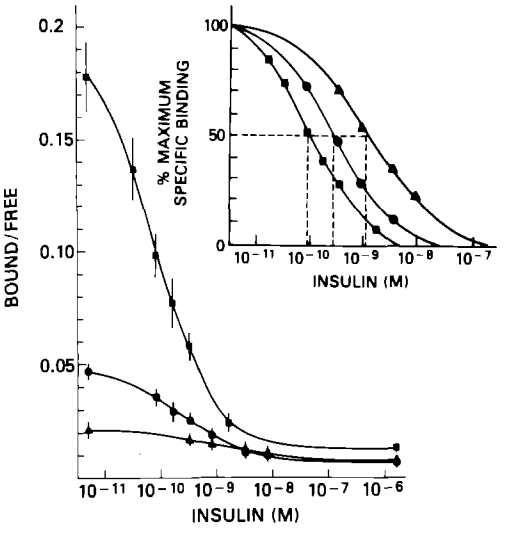

Figure 2. ^{125}I-Insulin binding to CHO cells. Wild type (●), B4-2-1 (■), and Lec 1.3c (▲) cells were prepared and processed as described in Methods. Bound/Free ratio normalized to mg cell protein is plotted as a function of the insulin concentration added. Identical curves were obtained if B/F ratios were corrected to DNA content. Points represent the mean of at least 6 exp ± SEM. Inset. The data have been normalized to % maximum specific binding. Dashed lines indicated the 1/2 Bmax concentrations. These data are summarized in Table 1.

1 µg/ml unlabelled insulin. Dissociation experiments on suspended cells using the DeMeyts technique (11) showed an accelerated dissociation of bound ^{125}I-insulin in the presence of 1 µg/ml insulin compared to dilution in buffer alone (data not shown). Thus by all criteria, wild type CHO cells express bona fide insulin receptors.

Finally, we measured the binding of ^{125}I-insulin to the two glycosylation mutants. As can be seen in Fig. 2, B4-2-1 cells bound four times as much ^{125}I-insulin as the wild type cells with a concomitant decrease in the concentration of hormone required for half-maximal displacement to 1.5×10^{-10}M. Therefore, B4-2-1 cell insulin binding is of higher affinity than that seen in wild type cells. Table I summarizes these and subsequent data. Lec 1.3c cells bound only half as much ^{125}I-insulin as the wild type cells with a half maximal displacement of 1.5×10^{-9}M. The affinity of the Lec 1.3c insulin receptor therefore appears to be lower than the wild type cells.

Discussion

The carbohydrate moeities of the insulin receptor have been examined by measuring insulin binding or bioactivity following digestion of cells with enzymes (3,4,5,), by blocking the glycosylation of newly synthesized proteins with inhibitors (5), or by biosynthetically labelling the receptor (2). The data presented in this paper confirm that alterations in the carbohydrate portion of the insulin receptor or adjacent molecules can have major effects on insulin binding and more specifically on receptor affinity. In the case of the Lec 1.3c cells, the carbohydrate defect is associated with the altered mobility of the α subunit of the insulin receptor on $NaDodSO_4$ gels (manuscript in preparation).

The abnormal processing of oligosaccharides in these cells is pleiotypic, i.e. it affects the synthesis of all glycoproteins. Thus, we cannot as yet say that the binding differences seen here are due to altered carbohydrate moieties on the insulin receptor subunits per se. It is possible that other membrane proteins such as the affinity regulatory component (12) or even adjacent molecules, either glycoproteins or glycolipids, able to interact with the insulin receptor, are involved in the altered binding. Since the change seen in insulin binding to its receptors in these mutants is one of affinity, studies are now in progress to determine the biological consequence of abnormal receptor affinity in these cell lines.

The CHO cell represents a system that is more amenable to manipulation than cultured human fibroblasts, more bioresponsive than freshly isolated blood cells, and more genetically defined as to specific carbohydrate abnormalities than any other cultured cell system. We have just begun to explore the numerous mutants available from CHO cells, but are encouraged by the dramatic findings represented in this report. By analyzing the binding to and the carbohydrates of the insulin receptor in these cells, we hope to gain valuable insights into the contribution of carbohydrates to the structure and function of the insulin receptor.

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