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Increased Surface Binding Sites of Insulin in ML236B (Compactin)-Resistant Mutants of Chinese Hamster Cell Line

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SUMMARY: Mutants resistant to ML236B (compactin) were isolated from the Chinese hamster lung V79 cell line (1). Three ML236B-resistant mutants, MF-1, MF-2 and MF-3, were enhanced in insulin-specific binding activity about 2 to 3 times over the parental V79 cell lines. Compared to V79, endocytosis of insulin was also increased 2 to 3-fold in ML236B-resistant mutants than V79. Scatchard analysis showed that 5,000 insulin binding sites per cell in V79 and 16,000 in a NL236B-resistant clone, MF-2. Insulin receptors in mutant and parental strains are down-regulated to a similar extent in the parental V79 treated with an excess insulin. This is the first somatic cell mutant with increased surface binding sites for insulin.

Various ligands including nutrients, plasma proteins, hormones, viruses and toxins bind to specific receptors on the cell surface and are then internalized by endocytosis (2-5). In particular, peptide hormones recognize specific receptors of target cells and the internalized hormones are sequestered into a variety of intracellular organelles such as lysosomes, Golgi, endoplasmic reticulum and the nucleus (5, 6).

Somatic cell mutants with altered receptors and endocytosis can help to understand the complex sequence of those events in cells. Mutants deficient in the binding and uptake of lysosomal hydrolases (7, 8), insulin (9, 10), low-density-lipoprotein (LDL) (11), ricin (12, 13) and other ligands have been isolated. Chinese hamster ovary (CHO) cell lines resistant to diphtheria toxin were found to show a pleiotropic defect in endocytosis of Sindbis virus (8) or a decreased number of binding sites for Pseudomonas exotoxin A (15).

Our Chinese hamster V79 cell mutants resistant to compactin (ML236B), a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are defective in endocytosis of LDL and resulted in increased sterol synthesis (1, 14). Here we have determined that ML236B-resistant ($ML236B^r$) mutants are

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not solely modified in LDL endocytosis (1) but show altered receptor levels and endocytosis activity for insulin.

MATERIALS AND METHODS

Cell lines in culture: The Chinese hamster cell line V79 and various ML236B^r mutants (MF-1, MF-2 and MF-3) were grown in monolayers in MEM containing 0.1% Bactopeptone (Difco Laboratory, Detroit, Mich.), 10% newborn calf serum (Flow Laboratory, Alexandria, Va.), kanamycin (100 ug/ml) and penicillin G (200 U/ml) as described previously (1, 16).

Chemicals and isotopic compound

Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Tokyo. Insulin (Porcine monocomponent, Novo Ind., A/S Denmark). [^{125}I]insulin (190 uCi/ug) (Dainabot Radioisotope Lab., Tokyo) was used.

Binding and internalization assays for insulin

Binding and internalization of insulin were assayed according to Olefsky and Kao (17). Binding was assayed in a CO_2-incubator with duplicate 35 mm dishes containing 1 ml of MEM + 0.1% bovine serum albumin. Following incubation with tracer amounts of [^{125}I]insulin at 4°C or at 37°C, cell cultures were washed 5 times with phosphate-buffered saline (PBS) at 4°C, and cultures were then incubated with 0.7 ml of 0.2 M acetic acid containing 0.5 M NaCl for 5 min at 4°C. The radioactivity soluble in acid was measured to assess binding activity. To measure the internalized activity, the dishes were further rinsed with 1 ml of cold PBS and the remaining cell-associated radioactivity was removed by incubation for 1 hr at 37°C in 1 N NaOH. The radioactivity removed by NaOH was measured to estimate the extent of internalization. Nonspecific binding and internalization were measured in the presence of 10 μg/ml of unlabeled insulin, and specific activities were determined after subtracting the nonspecific values.

Down-regulation of insulin receptors

Cells were exponentially grown for 18 hr in MEM containing 10% serum and were then further incubated for 4 hr or 8 hr in MEM containing 2% serum without or with 1 μg/ml or 5 μg/ml of unlabeled insulin. Binding assays were performed after a further incubation with labeled insulin for 60 min at 4°C.

Gel-filtration analysis by Sephadex G-50 column chromatography

Cell solubilization and subsequent chromatographic analysis were performed by the method of Gorden et al. (18). After incubation in MEM containing 0.1% bovine serum albumin and [^{125}I]insulin for 1 hr at 37°C, cultures were washed 5 times with cold PBS and solubilized in a solution containing 0.1% Triton X-100, 3 M acetic acid and 6 M urea. The solubilized material was then centrifuged for 5 min at 10,000 x g at 4°C and the supernatant (total bound and internalized activity) was applied to a 50 x 1.1 cm Sephadex G-50 column and eluted with 1 M acetic acid. The [^{125}I]insulin activity not extracted by acid (internalized activity) was also fractionated on the column.

RESULTS AND DISCUSSION

Three ML236B^r mutants (MF-1, -2, and -3) were found to be defective in endocytosis of low-density-lipoprotein (LDL), but showed LDL binding activities similar to the parental V79 (1). Using the acid-extraction technique of Olefsky and Kao (17), we compared binding and endocytosis activity of insulin for V79 and ML236B^r cells. Acid-extractable and acid-non-extractable activities are

Table 1. Comparison of Binding and Endocytosis of [^{125}I]Insulin in V79 and ML236B^{r} Clones

Cell Lines	Specific Activity per 10^6 Cells (%)		Specific Activity per mg Protein (%)	
	Binding	Endocytosis	Binding	Endocytosis
V79	0.05	0.22	0.45	2.1
MF-1	0.11	0.32	0.99	2.9
MF-2	0.20	0.57	1.8	5.2
MF-3	0.12	0.42	1.1	3.8

Monolayers of V79 and three ML236B^{r} mutants (MF-1, MF-2 and MF-3) were incubated with [^{125}I] insulin for 60 min at 37°C. The monolayers were then treated with acid to release the cell surface bound [^{125}I]radioactivity (Binding), and the remaining cells were treated with 1 N NaOH to release acid non-extractble radioacitivity (Endocytosis). Specific bound radioactivity per 10^6 cells and per one mg protein were represented as % of total input [^{125}I] insulin radioactivity. Data are average values from duplicate trials.

identified as cell surface bound activity and endocytosed activity respectively. More than 60-70% of total radioactivity is removed by an excess of unlabeled insulin (Table I) in each cell type. The binding activities of MF-1, -2 and -3 were then 2 to 4-fold higher than that of V79. The enhanced binding of insulin in three ML236B^{r} mutants was accompanied by enhanced endocytosis (Table I).

The kinetics of binding and endocytosis in V79 and a ML236B^{r} clone (MF-2) at 4°C and 37°C are shown in Fig. 1. Insulin can associate with the cell surface at low temperature, but no endocytosis occurs (16). At 4°C, MF-2 showed about 3-fold more binding than V79 (Fig. 1 A). Endocytosis then increase in V79 and MF-2 at 37°C (Fig. 1 B). At higher temperature, MF-2 still showed about 2-fold more surface binding and endocytosis of insulin (Fig. 1 B). The enhanced internalization in the ML236B^{r} clone is presumably secondary to enhanced binding activity.

To evaluate the fate of insulin internalized in V79 and MF-2, the total cell-associated radioactivity and non-extractable radioactivity were chromato-

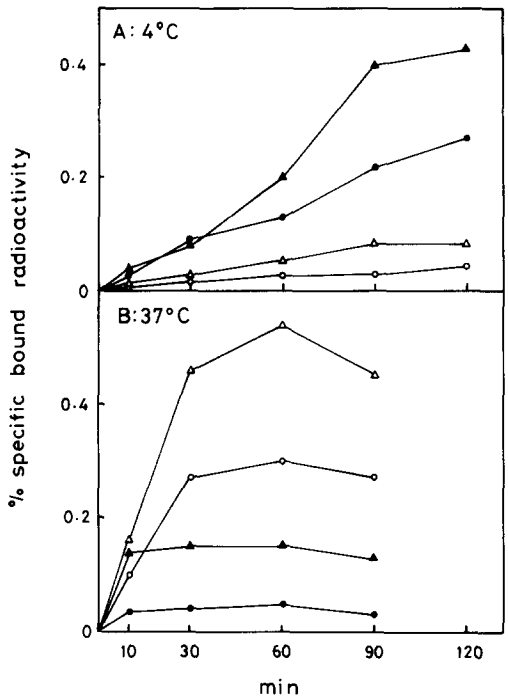

Fig.1. Time course of [^{125}I] insulin binding and internalization at 4°C (A) and at 37°C (B). Monolayers of V79 cells and MF-2 cells were incubated with [^{125}I]insulin for the indicated time in the absence or presence of 10 μg/ml of unlabeled insulin. At each point, monolayers were treated with acid to release the cell surface bound radioactivity, and the remaining cells were measured for the internalization after lysis with NaOH. Data are average values from duplicate samples and specific bound or internalized radioacitivity (%) per 10^6 cells was divided by total input [^{125}I] radioactivity. Symbols:bound activity in V79 (●) and MF-2 (▲); internalized activity in V79 (○) and MF-2 (△).

graphed on Sephadex G-50. The elution profile in Fig.2 shows three peaks of radioactivity when V79 or MF-2 cell from [^{125}I]insulin extracted after 60 min at 37°C. About 80% of the total cell-associated radioactivity is internalized in both V79 and MF-2 cells. In both cell lines, only small amounts of degradation products (peak III) were observed. About twice the radioactivity was observed in MF-2 than in V79 for both the cell-associated and internalized fractions (Fig. 2). Intracellular insulin is thought to be degraded through lysosomal

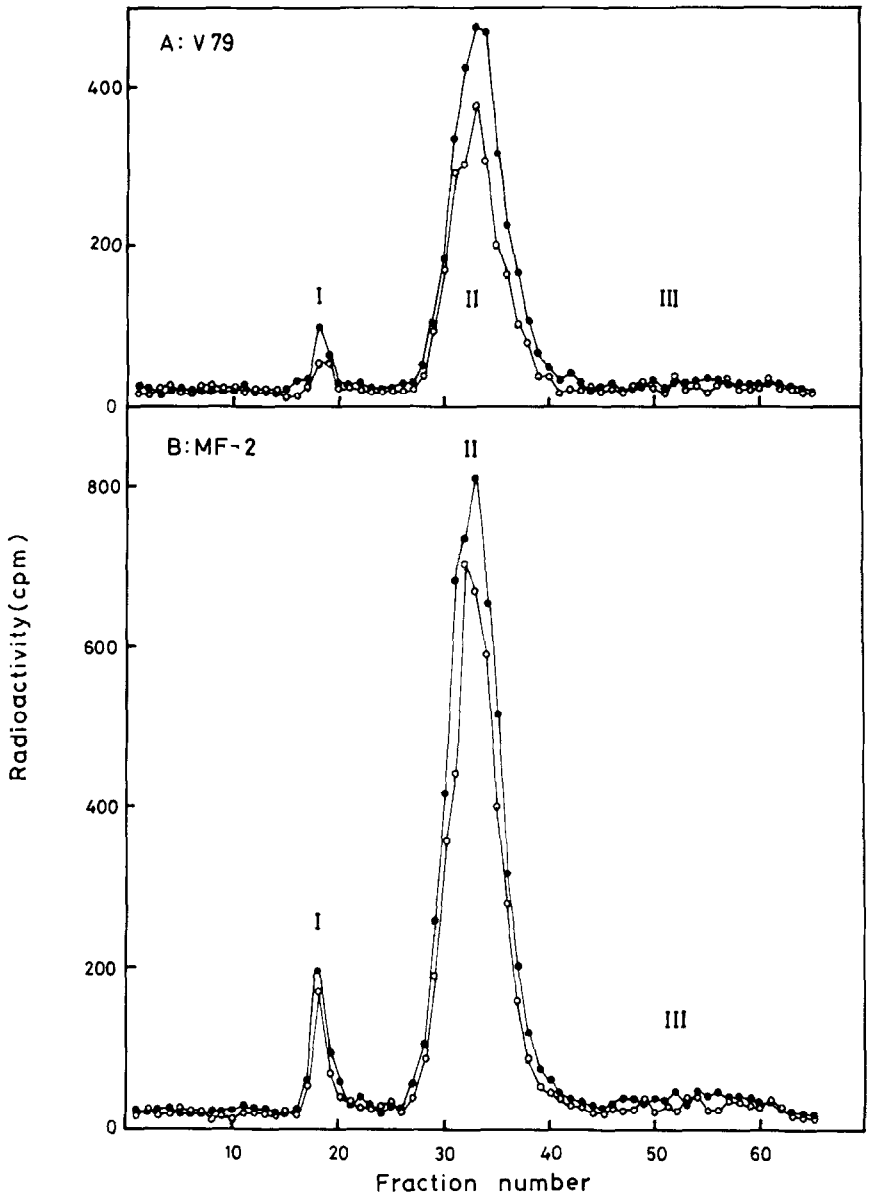

Fig.2. Sephadex G-50 profiles of total and acid non-extractable radioacitivity. Monolayeres of V79 (A) and MF-2 (B) cells were incubated with [^{125}I]insulin for 60 min at 37°C , and solubilized in 0.1% Triton X-100, 3M acetic acid and 6M urea. The solubilized material was applied to a Sephadex G-50 column. Symbols: elution profile of total bound activity (●) and of internalized activity (○). The [^{125}I] radioactivity at peak I at the void volume, presumably represents an aggregated form of insulin; peak II represents intact [^{125}I]insulin; peak III represents degradation form of [^{125}I]insulin.

pathway after the fusion of endocytotic vesicles (19); and since comparable low amounts of degradation products of insulin were observed in both V79 and MF-2 (Fig. 2), altered lysosomal function in the resistant clone does not seem to be involved in enhanced uptake.

To ascertain whether the enhanced insulin-binding activity reflected a change in the absolute number or affinity of receptors, [^{125}I]insulin binding was assayed as a function of insulin dose. V79 and MF-2 cells were incubated with [^{125}I]insulin at 4°C for 120min and the influence of unlabeled insulin concentration on cell-associated radioactivity is illustrated in the Scatchard plots of Fig. 3. The overall slopes of the curves were found to be complex (20), but they are similar for the two cell lines. This indicates a change in the receptor number with little change in the receptor affinity for insulin for V79 and MF-2 (Fig. 3). V79 and MF-2 cells have respectively about 5,000 and 16,000 insulin receptors. Exposure of cultured cells to insulin has been shown to induce receptor down-regulation in various cell lines (21-23). We compared the capacity of V79 and MF-2 cells to down-regulate cellular level of insulin

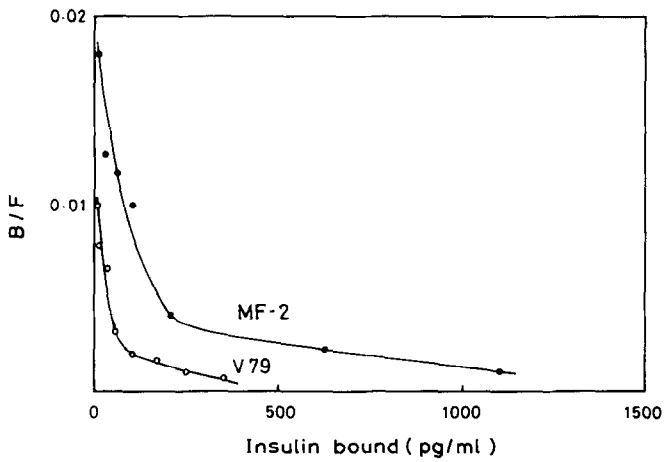

Fig.3. Binding characteristics of insulin receptors at 4°C. Monolayers of V79 cell (○) and MF-2 cell (●) were incubated with 1.5 ng/ml of [^{125}I]-insulin and various doses of unlabeled insulin at 4°C for 120min. A competition for binding of [^{125}I]insulin by unlabeled insulin was drawn and Scatchard plot analysis of the data was presented.

Table II. Insulin-Induced Down-Regulation of Insulin Receptor

Cell Lines	Insulin (μg/ml)	Receptor Activity of Control (%)	
		4 hr	8 hr
V79	1	48.6	44.9
V79	5	14.6	21.5
MF-2	1	51.9	34.6
MF-2	5	13.7	20.8

Monolayers of V79 and MF-2 were incubated in MEM medium containing 10% NCS with or without 1 and 5 μg/ml of insulin. After incubation with cold insulin for 4 hr or for 8 hr, insulin binding assay was performed at 4°C for 2 hr. Per cent of control at each point was presented. Data are average values of duplicate trials.

receptor upon addition of insulin to the medium. Both cell lines were cultured for 4 hr or 8 hr with 1 μg/ml of insulin or 5 μg/ml of insulin and were then assayed for their insulin binding activity. In both V79 and MF-2 cells, [^{125}I]-insulin binding activity at 4°C for 120min was reduced to about 50% of the control after a 4 hr or 8 hr incubation with 1 μg/ml of insulin (Table II). The receptors were comparably down-regulated to about 20% of the control after incubation with 5 μg/ml of insulin (Table II). Thus, down-regulation seems unaltered in MF-2 compared to V79.

The major change in the response of ML236B^r cells to insulin is thus one in the level of receptors. This suggests that the binding of one ligand or its extent of endocytosis may regulate the receptor and endocytosis activity of cells to other ligands: changes in one produce pleiotropic changes in the other. The level at which such a regulation might be exerted is not known. One possibility is that receptor number and endocytosis are regulated by the level of a common protein or step. For example two unrelated ligands sometimes exist in the same coated vesicles: epidermal growth factor and transferrin (24) or epidermal growth factor and adenovirus 12 (25). Ray and Wu (12) isolated CHO

mutants simultaneously resistant to ricin and Pseudomonas toxin which were defective in endocytosis of both toxins.

Alternatively, because the functions of the membrane are complex and interdependent, the initial change in a mutant may affect a number of other phenotypes, leading to adjustments in levels of membrane components, with different adjustments altering the response to different agents. Thus the pleiotropy in ML236B^r is extreme; and in particular, we show here that it can involve not only lowered levels of various receptors, as observed in a number of cases such as LDL (1), ricin or latex beads (unpublished data), but also the first known instance of an increased number of insulin receptors. Understanding the basis for the increase may require a knowledge of the primary site of the mutation.

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