Cell-Cycle Analysis of Insulin Binding and Internalization on Mouse Melanoma Cells

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ABSTRACT Binding of ^{125}I-labeled insulin to the surface receptors of Cloudman S-91 mouse melanoma cells (CCL 53.1) was studied at various phases (M, G_1, S, and G_2) in the cell cycle. Insulin-binding activity was persistently present during the cell cycle but the highest activity was noted at the S-phase. The insulin once bound to the cell surface receptors at any phase of the cell cycle was internalized and degraded, presumably through a lysosomal pathway. Insulin-binding activity of melanoma cells was not affected by melanocyte-stimulating hormone.

Among the polypeptide hormones, insulin is one of the most complex in action because it plays diverse functions in the regulation of carbohydrate, amino acid, and fat metabolism (6, 21). Insulin is also known to stimulate DNA synthesis and promote cellular growth in many cell types in culture through modulating a number of cellular processes (14, 17, 30). These effects appear to be triggered by its interaction with cell surface glycoprotein receptors (3, 18, 19, 25, 28). A number of biochemical parameters for insulin/receptor interaction have been characterized and compared in terms of properties of various cells, such as growing and quiescent cells, normal and transformed cells, and differentiating and nondifferentiating cells (2, 13, 24, 27). Furthermore, recent studies have indicated that some form of compartmentalization or internalization occurs during insulin's interaction with the receptor and that the receptor-bound insulin undergoes a substantial degradation or processing that is associated with subcellular organelles (1, 9, 10, 16). Insulin has recently been shown to alter proliferation rates in Cloudman S-91 mouse melanoma cells in culture, presumably by altering intracellular levels of cyclic AMP (20).

In this paper we describe the binding of insulin to cellular receptors and the fate of cell-bound insulin at various phases of the cell cycle in the synchronized cell populations of Cloudman S-91 melanoma.

MATERIALS AND METHODS

Cell Cultures

Cloudman S-91 mouse melanoma cells (Clone M-3, CCL 53.1) were obtained from the American Type Culture Collection. Cells were grown in Ham's F10 nutrient medium supplemented with 2% fetal calf serum, 10% horse serum (Flow Laboratories, Inc., Rockville, Md.) and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml; Microbiological Associates, Walkersville, Md.) under 5% CO_2/95% air at 37°C. Cells were synchronized by the mitotic shakeoff method described previously (8). In this procedure no coercive agents such as colchicine are used to restrict cell-cycle traverse. Melanocyte-stimulating hormone (MSH) was prepared in the laboratory of Dr. Victor Hruby and kindly provided to us.

Assay for ^{125}I-labeled Insulin Binding

Cells grown in 60-mm-diameter plastic culture dishes were placed on ice and washed with ice-cold binding (B)-buffer, 2 ml each. The B-buffer contained 100 mM HEPES (Sigma Chemical Co., St. Louis, Mo.), pH 7.8, 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 10 mM glucose, 1 mM EDTA, 15 mM Na-acetate and 1% bovine serum albumin (Sigma Chemical Co.; RIA grade). These stock solutions were kept frozen until use. 1 ml of B-buffer containing 1×10^{-10} M (or 1×10^{-9} M) ^{125}I-labeled porcine insulin (New England Nuclear, Boston, Mass.; 100 μCi/μg) was added and the cells were incubated at 15° or 37°C for various times. The plates were washed three times with ice-cold B-buffer, 3 ml each. The cells were then solubilized with 1 ml of 0.5 N NaOH. Aliquots (0.9 ml) were mixed with 7 ml each of Riafluor (New England Nuclear) and acidified with 75 μl of 6 N HCl. Radioactivity was counted in a Packard liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). To measure nonspecific binding, 1 μl of unlabeled porcine insulin (1 mg/ml of 0.01 N HCl, 26.6 U/mg; Elanco Products Co., Indianapolis, Ind.) was added immediately before the addition of radioactive insulin. Nonspecific binding thus measured ranged from 10 to 15%, which was subtracted from the total binding to estimate specific binding.

Location of Cell-bound Insulin

This was analyzed by the following two methods: (*a*) Cell cultures were incubated with ^{125}I-insulin under the conditions described in the previous paragraph. After incubation at 37°C for various times, the cells were placed on ice, washed three times with ice-cold B-buffer, and then treated with 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl at 4°C for 6 min to remove cell-surface bound ^{125}I-insulin (11). The remaining ^{125}I-insulin that had been compartmentalized or internalized was solubilized in 1 N NaOH. (*b*) The cells that had been treated with ^{125}I-insulin and washed extensively with B-buffer at 4°C were dissolved in 0.4 ml of a solution consisting of 4 M urea, 1 M acetic acid and 0.1% Triton X-100. The cell lysate was clarified by centrifugation at 30,000 *g* for 30 min. The supernate was loaded onto the top of a column (0.9 × 28 cm) of Sephadex G-75 that had been equilibrated with the same solution (16, 26). Fractions of 0.4 ml each were collected, and radioactivity was counted as before. Recovery of the cell-bound radioactivity in the effluent of the column ranged from 95 to 100%. The void volume and the elution position of native insulin were determined by a separate running with blue dextran 2000 and ^{125}I-insulin, respectively.

To test the involvement of lysosomal proteases in insulin degradation, cells were incubated with 0.1 mM chloroquine for 20 min at 23°C before insulin binding assay. In this case, the B-buffer contained 0.1 mM chloroquine. To remove surface-bound ^{125}I-insulin, cells were incubated with trypsin (1 mg/ml) in B-buffer for 5 min at 37°C.

Cell Counting and Size Analysis

Cells were removed from the culture dishes by conventional trypsinization and counted by Electrozone/Celloscope (Particle Data, Inc., Elmhurst, Ill.). Counting variations were 5–10% on duplicate dishes, and the frequency of live cells was usually 95–100%, which was determined by the trypan blue dye exclusion test. Cell size distribution and mean volume estimation were carried out according to the Particle Data instruction manual, using standard polystyrene beads (Duke Scientific Corp., Palo Alto, Calif.).

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RESULTS

Fig. 1 *A* shows the time-course of binding of ^{125}I-insulin to Cloudman S-91 mouse melanoma cells in monolayer at 15° and 37°C. At 15°C, maximal binding was reached after incubation for 60 min and the amount of cell-bound radioactivity remained constant, whereas at 37°C a rapid decrease of cell-bound radioactivity was observed after reaching a maximum at 10–30 min. This is a well-documented phenomenon in various cell types exposed to either insulin or other polypeptide hormones and has been attributed to the consequence of internalization followed by intracellular degradation and secretion (4, 7, 9, 15, 16). Fig. 1 *B* shows the distribution of radioactivity into the cell exterior and cell interior during the initial 30-min incubation at 37°C. For this, cells were incubated with ^{125}I-insulin, washed to remove unbound hormone as described in Fig. 1 *A*, and then treated with acetic acid (pH 2.5) containing 0.5 M NaCl at 4°C. The radioactivity released by this acid treatment is derived mainly from the cell surface, and the remaining acid-resistant counts are of intracellular origin, namely, internalized insulin, as is evidenced below. In Fig. 1 *B*, ^{125}I-insulin was initially found in an acid-soluble fraction but after 10 min there was a decrease in this fraction with a concomitant increase in the acid-resistant fraction, indicating a transition from acid solubility to acid resistance. Analysis of the acid-soluble fraction on Sephadex G-75 gel filtration (16, 26) revealed that >90% of the radioactivity eluted with native insulin. The acid-resistant fraction was found to be a mixture of iodotyrosine-containing oligopeptides and intact ^{125}I-insulin, of which the ratio (degraded/intact) increased with time. When cells that had been incubated with ^{125}I-insulin for 30 min were treated with trypsin, >90% of the acid-soluble counts were lost, whereas acid-resistant counts remained unchanged (Table I). Contrary to this, the acid-resistant counts were substantially reduced when cells were treated with chloroquine, a lysosomal protease inhibitor, for 20 min before insulin binding. It is noted that chloroquine treatment increased overall binding of insulin. All these data seem to support the idea that the insulin bound to the surface receptors of mouse melanoma cells undergoes internalization and is subject to intracellular degradative processing, perhaps through a lysosomal pathway. Approximately two-thirds of the cell-bound insulin is processed by 30 min at 37°C.

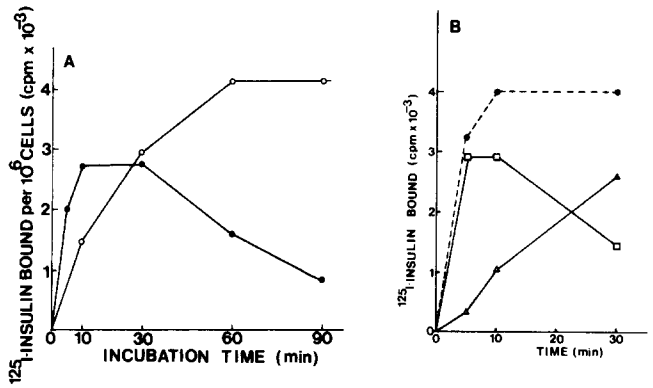

FIGURE 1 (*A*) Time-course of ^{125}I-insulin binding to mouse melanoma cells at 15° (○) and 37°C (●). ^{125}I-insulin concentration was 1×10^{-9} M. (*B*) Time-course of processing of cell-bound insulin. Cells were incubated with ^{125}I-insulin, at 37°C for the indicated time, washed to remove unbound hormone, and then treated with 0.2 M acetic acid/0.5 M NaCl. ●, Total; △, acid resistant; □, acid soluble.

TABLE I

Effects of Trypsin and Chloroquine on Insulin Binding and Processing

Treatment	^{125}I-insulin bound, *cpm/dish*		
	Total binding	Acid-soluble	Acid-resistant
None (control)	4,020	1,440	2,580
Trypsin*	2,339	86	2,253
Chloroquine‡	4,830	3,910	920

Cloudman S-91 melanoma cells were incubated with 1×10^{-9} M ^{125}I-insulin at 37°C for 30 min.

* Insulin-treated cells were incubated with trypsin (1 mg/ml) for 5 min at 37°C.

‡ Cells were treated with chloroquine (0.1 mM) for 20 min at 23°C before insulin-binding assay.

To the best of our knowledge, insulin-binding activity has not been reported in terms of the cell cycle. Because Cloudman S-91 melanoma cells are easily synchronized by mitotic shakeoff (8) and because these cells exhibit reasonably high activity of insulin binding (Fig. 1 and Table I), we undertook the cell-cycle analysis of insulin binding to surface receptors. In these experiments, mitotic cells were inoculated into fresh medium, and insulin binding was measured at various times. We have previously shown that the length of the Cloudman mouse melanoma cell cycle is 24 h and that the lengths of the cell-cycle phases are: G_1, 10.0 h; S, 8.0 h; G_2, 2.0 h; and M, 4.0 h (8). The time intervals used to measure insulin binding were thus picked to coincide with these cell-cycle phases. The results in Table II clearly show that insulin receptors are persistently present during the cell cycle. It is noted, however, that cells at S phase exhibit the highest insulin-binding activity, which is two to four times higher than in G_1-phase cells (note relative activity shown in parentheses in Table II). The difference in insulin-binding activity is more accurately demonstrated when cell surface area (estimated from cell-volume analysis) is considered. It should be noted that cells 26 h after mitotic shakeoff, namely in second cycle of G_1, possess insulin-binding activity similar to that in the first G_1 phase. This finding eliminates the possibility that insulin in serum may have masked receptors during the initial G_1 period after inoculation and inhibited ^{125}I-insulin binding until it was exhausted from the medium.

It has recently been shown that insulin and MSH act antagonistically to control cell proliferation of Cloudman S-91 melanoma cells (20). Therefore, we examined the possibility that MSH binding to cellular receptors might modulate the levels of available insulin receptors. As is shown in Table III, insulin-binding activity was not influenced by pretreatment of the cells with MSH for three time periods up to 24 h.

Next, we analyzed the fate of the cell-bound insulin during the cell cycle. For this, cells treated with ^{125}I-insulin at 37°C for various times were washed extensively with binding buffer to remove unbound insulin, dissolved in a solution containing urea, acetic acid, and Triton X-100, and subjected to gel filtration on Sephadex G-75. Previous studies in which similar conditions were used have revealed the presence of intact insulin and two new radioactive components, one with a smaller and one with a larger molecular weight than that of insulin (15, 23, 25). Fig. 2 shows elution profiles of radioactivity resulting from the ^{125}I-insulin bound to the melanoma cells that are at G_1, S, and G_2, respectively. The majority of cell-bound radioactivity eluted at the position corresponding to the insulin marker (peak *II*) at 5 and 10 min. This peak was greatest for every phase at 10 min and declined at 30 min. The

TABLE II

Insulin-binding Activity of Mouse Melanoma Cells at Various Phases of Cell Cycle

Time after mitotic shakeoff	Corresponding phases	Insulin-binding ability, % of input counts/10^6 cells				Relative cell volume*	Insulin-binding ability per cell surface‡
		Exp. I	Exp. II	Exp. III	Average		
h							
0	M	nt	nt	0.67 (0.7)	0.7	nt	
4.5	G_1	0.57 (1.0)	0.71 (1.0)	0.91 (1.0)	1.0	1.0	1.0
14	S	1.38 (2.4)	2.82 (4.0)	2.44 (2.6)	3.0	1.6	2.2
19	G_2	0.64 (1.1)	1.10 (1.6)	2.01 (2.2)	1.6	1.6	1.2
26	G_1 (2nd)	0.54 (1.0)	0.44 (0.6)	1.71 (1.9)	1.2	1.3	1.0

Cells were incubated at 15°C for 60 min with 1×10^{-9} M ^{125}I-insulin. For details, see Materials and Methods. *nt*, not tested.

* Mean cell volume was determined by an Electrozone/Celloscope (see Materials and Methods).

‡ Average of insulin-binding activity was normalized to cell surface area, which was calculated from the mean cell volume.

TABLE III

Insulin-binding Activity of Mouse Melanoma Cells after Treatment with MSH

Preincubation with MSH	Insulin binding, % of input counts/10^6 cells		
	With MSH (A)	Without MSH (B)	Ratio (A/B)
h			
2	0.72	0.68	1.05
7	0.76	0.67	1.14
24	0.70	0.67	1.07

Cells were grown in the presence of 1×10^{-7} M MSH for the time indicated and then incubated with 1×10^{-9} M ^{125}I-insulin at 15°C for 60 min under the conditions of the binding assay.

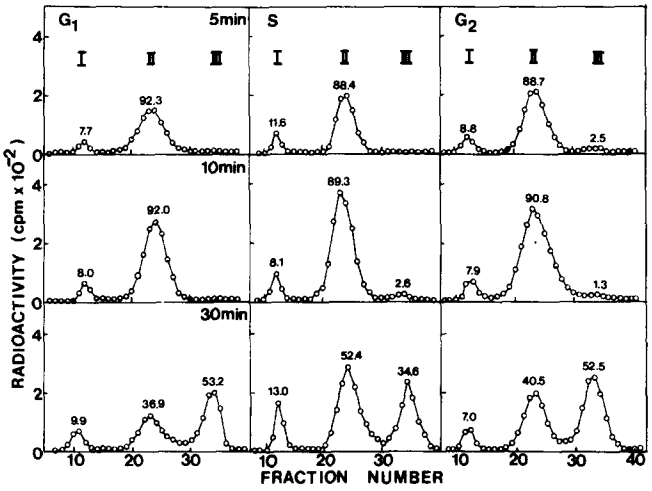

FIGURE 2 Gel-filtration patterns of ^{125}I-insulin bound to mouse melanoma cells at the indicated cell phase. Cells were incubated at 37°C with ^{125}I-insulin (1×10^{-9} M) as described under Materials and Methods. At the indicated times, cells were washed with ice-cold buffer to remove unbound insulin, dissolved in 4 M urea, 1 M acetic acid, and 0.1% Triton X-100, and subjected to gel filtration on Sephadex G-75. Void volume was fraction *12*; elution position of ^{125}I-insulin was fraction *24*; total column volume was fraction *35*. Distribution of the cell-bound ^{125}I-insulin into peaks *I*, *II*, and *III* is indicated as percent of the total radioactivity recovered.

radioactive component that eluted just before the position of the total column volume (peak *III*) corresponds to the iodotyrosine-containing oligopeptides (16, 26), which must be of intracellular origin, because (*a*) otherwise this would have been removed during extensive washing on ice, (*b*) the material in this peak *III* was accumulated during 10–30 min, whereas intact insulin in peak *II* decreased, (*c*) the peak *III* materials were the major component found in the insulin-treated cells that had been incubated with trypsin to remove surface-bound insulin in a manner similar to that in Table I, (*d*) the appearance of peak *III* has been shown to be substantially reduced after treatment of cells with inhibitors of lysosomal proteases, such as chloroquine. A significant fraction of the cell-bound radioactivity is processed as a high molecular weight component that elutes at the void-volume position (peak *I*). This has been observed by us and others (16, 26) and is postulated to be insulin irreversibly bound to cellular components including insulin receptors. It is interesting to note that peak *I* material is accumulated more in the cells at S phase at 30 min. Taking all these data together, we conclude that an internalization of the cell-bound insulin and its processing, perhaps through the lysosomal degradation pathway, occurs during the entire cell cycle.

DISCUSSION

The investigation of peptide hormone binding to surface receptors throughout the cell cycle has received little attention. The appearance of surface receptors for nerve growth factor in neuroblastoma cells is confined to the G_1-S border (22). Receptors for MSH are expressed only in the G_2 phase (28), and the stimulation of tyrosinase in these cells by MSH is similarly confined to the G_2 phase (29). More recent studies, however, have shown that MSH can induce tyrosinase activity in any phase of the cell cycle, a finding that suggests that MSH receptors are present throughout the cell cycle (8).

The results presented here have not only demonstrated the presence of insulin receptors in Cloudman S-91 melanoma cell cultures but have shown that the level of expression of these surface receptors is regulated throughout the cell cycle. Insulin is required for the growth of one line (M2R) of mouse melanoma cells in a chemically defined medium (12). However, it has been observed that insulin concentration as low as 10^{-10} M can retard growth rates of Cloudman S-91 melanoma cell cultures (20). Concentrations of 10^{-8} M were reported to arrest proliferation completely (20). It was suggested that insulin exerts its inhibitory effects by lowering cyclic AMP levels (20). Because the inhibitory effect of insulin on cell growth was shown to be antagonized by exposure of insulin-treated cells to MSH (20), we investigated the effect of MSH on insulin binding. We noted, however, no change in binding capacity. The data at 2-h incubation (Table III) may exclude the possibility that loss of insulin receptors is transient and is promptly recovered to the steady-state level. Because insulin apparently lowers cyclic AMP levels and suppresses, to some extent, the

stimulation of cyclic AMP production by MSH, it is possible that insulin lowers the level of expressed MSH receptors. It is also possible that insulin action in melanoma cells proceeds by a mechanism distal to the initial hormone-surface receptor binding event.

Recent evidence suggests a possible role for peptide hormone internalization in mediating various cellular responses (1, 4, 16). We have noted here that insulin is internalized in melanoma cells, and our results suggest that the hormone begins to undergo degradation within 30 min after receptor binding. It is therefore possible that, before degradation, insulin exerts its cellular effects or that even after 30 min enough intact hormone is available to effect the cellular response, or that degradation products function as a second messenger.

Our finding that the level of expression of insulin receptors is regulated throughout the cell cycle, being highest at S and lowest during G_1 phase, suggests a possible role for cell-cycle traverse in regulating hormonal responsiveness. This observed difference in insulin binding during the cell cycle could be physiologically important only if the level of binding in G_1 is insufficient to maximally affect the cell growth. The reported presence of "spare receptors" for insulin as well as for other peptide hormones suggests that a maximal cellular response can occur without maximal receptor binding (4, 5, 15, 23). Thus, if the melanoma cell response to insulin is maximal in G_1 cells, then the importance of the increased level of hormone binding in S to cellular regulation is questionable. It would, however, be important to study whether increase of insulin binding at S phase occurs because of a change in the number of receptors or in a receptor's affinity.

We have recently observed that exposure of melanoma cells to insulin results in a decrease not only in basal tyrosinase activity but also in the level of MSH-induced enzyme activity (our unpublished observation). Studies are now in progress to determine whether this inhibitory effect of insulin occurs throughout the cell cycle or is confined to a particular phase, for example, S.

The possibility that the presence of functional receptors at particular phases of the cell cycle represents another regulatory control point for the cellular response to hormonal stimulation remains to be investigated.

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