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Photoaffinity Labeling of the Insulin Receptor in H4 Hepatoma Cells: Lack of Cellular Receptor Processing

Cecilia Hofmann, Tae H. Ji, Bonnie Miller, and Donald F. Steiner

Department of Biochemistry, The University of Chicago, Chicago, Illinois 60637 (C.H., B.M., D.F.S.), and Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071 (T.H.J.)

Photoaffinity labeling techniques were used to identify insulin-binding components of the plasma membrane in insulin-responsive, monolayer-cultured hepatoma cells. The activated, photosensitive reagent, an n-hydroxysuccinimide ester of 4-azidobenzoic acid, was coupled with highly purifed insulin, and the hormone derivative was subsequently iodinated, bound to cell surface receptors of intact H4 cells, and photoactivated. After dissolution of the cells, labeled proteins were analyzed by SDS/polyacrylamide gel electrophoresis under reducing conditions. The main labeled band exhibited an apparent molecular weight of 130,000. Two minor components of apparent mol wt 95,000 and 40,000 were also identified. Specific labeling of all 3 bands was inhibited by simultaneous incubation of the cells with native insulin, but not by the heterologous hormone, glucagon, prior to photoactivation. Binding of azidobenzoyl-insulin to H4 cells was time-dependent, as was the correlated labeling of receptor components. Band-labeling by the photosensitive insulin derivative was totally light-dependent; spontaneous covalent linking of insulin and receptor was not observed. The labeled receptor-related proteins were not degraded by the cells under our experimental conditions.

Key words: insulin receptors, photoaffinity labeling, electrophoresis

The initial event leading to the biological effects of insulin in target cells is generally considered to be hormone binding to plasma membrane receptors [1–3]. Following hormone binding, aggregation of receptors occurs [4–6], succeeded by endocytotic uptake of the hormone-receptor complex leading to lysosome-mediated insulin degradation [7–9] and extracellular release of hormonal degradation products [7, 8, 10]. Although these events are probably important in the metabolism of insulin in vivo [8], their role(s) in mediating cellular responses to insulin remains unknown. To identify receptor components directly and to examine their intracellular metabolic fate following hormone binding in intact cells, we have synthesized an activated heterobifunctional cross-linking reagent, the n-hydroxy-succinimide ester of 4-azidobenzoic acid, which can be coupled to insulin

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through its free amino groups. The radiolabeled hormone derivative was then covalently linked to receptor components of cultured hepatoma cells via light-induced conversion of the aryl azide moiety to a highly reactive aryl nitrene.

In this report, we describe the specific labeling of 3 high-molecular weight receptor-related proteins in intact, biologically responsive, hepatoma cells using the radioactive, photoreactive insulin derivative. Following solubilization of labeled cells, SDS polyacrylamide gel electrophoresis under reducing conditions showed labeling of a major band having a mol wt of about 130,000 and 2 minor bands of 95,000 and 40,000. These receptor-related proteins were not degraded in the cells during postincubation conditions.

MATERIALS AND METHODS

Cell Cultures

The H4-II-E-C_3 hepatoma cells [11] used for this study were supplied by Dr. Alan Horwitz, Department of Pediatrics, The University of Chicago, and were continuously cultured as monolayers as described previously [12].

Reagents

Monocomponent porcine insulin was a generous gift of Novo Research Laboratories (Copenhagen), and glucagon was kindly provided by Dr. Howard S. Tager, Department of Biochemistry, The University of Chicago.

Cross-Linking Reagent Preparation

The N-hydroxysuccinimide ester of 4-azidobenzoic acid (NHS-ABA) was prepared by conversion of 4-aminobenzoic acid to 4-azidobenzoic acid [13], which in turn was treated with N-hydroxysuccinimide and dicyclohexylcarbodiimide. N-hydroxysuccinimide (41 mmoles in 15 ml of dry dioxane) and dicyclohexylcarbodiimide (42 mmoles in 10 ml of dry dioxane) were introduced sequentially into 40 mmoles of 4-azidobenzoic acid dissolved in 40 ml of dry dioxane. This mixture was stirred for 5 h and the precipitate recovered by filtration. The filtrate was evaporated to dryness and the resulting powder washed with boiling petroleum ether to remove unreacted N-hydroxysuccinimide.

ABA Derivatization of Insulin

NHS-ABA was freshly dissolved in dimethylformamide to a concentration of 200 mM and was added to a 1-mg/ml solution of insulin (in phosphate-buffered saline (pH 7.4)–50% dimethylformamide) to a final concentration of 1, 2, 5, or 10 mM reagent. The mixture was incubated in the dark for 60 min at 23°C with occasional mixing and then stored at −20°C.

Iodination of ABA Insulin

Derivatized insulin was labeled with $Na^{125}I$ to a specific activity of 50–100 μCi/μg by a modification [14] of the method of Freychet et al [15].

ABA-^{125}I-Insulin Binding and Cross-Linking to H4 Cells

Confluent monolayer cultures of H4 cells (1–4 × 10^6 cells/60-mm plate) were washed with Hank's buffer and incubated with 1.5 ml. Swim's S-77 medium containing 20 mM HEPES, 5 mM sodium bicarbonate, and 1% bovine serum albumin, pH 7.4. ABA-^{125}I-insulin

was added to a final concentration of about 3×10^{-9} M under dark conditions. Unless specified otherwise, binding was carried out at 15°C for 2 h in the dark. Cells were then rinsed 8 times with 2 ml ice-cold Hank's buffer, and photoactivated cross-linking of bound ABA-^{125}I-insulin was induced by a 5-min irradiation of the cell monolayer (in 2 ml Hank's buffer) with a UVS-11 Mineralight (Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 1.5 cm. The cell monolayer was then rinsed, solubilized in 1.5 ml of 88% formic acid, and transferred to counting tubes to determine the amount of radioactivity bound. Samples were then dried under vacuum and stored at −20°C. Prior to electrophoresis, samples were solubilized in appropriate buffers as described below.

Electrophoresis and Autoradiography

Samples of ABA-^{125}I-insulin were solubilized in 1 M acetic acid containing 8 M urea and were run on pH 4.5 polyacrylamide gels in 8 M urea [16] to determine the extent of derivatization by the reagent. Migration of derivatives was evaluated by counting 1.5-mm gel slices in a Gamma scintillation spectrometer (Packard Instrument Company, Downers Grove, IL).

Evaluation of the photolinked insulin-receptor complex was made by running cell samples prepared as described previously on discontinuous slab gels according to the technique of Laemmli et al [17]. Samples were solubilized in 0.0625 M Tris (pH 6.8), 2% SDS, 0.001% bromophenol blue, 8 M urea, 5% mercaptoethanol and boiled for 3 min prior to electrophoresis at 30 mamp for 4–5 h on 1-mm-thick gels containing 5.0%, 7.5%, 10.0%, or 15.0% acrylamide with 3.0% or 5.0% stacking gels.

Gels were stained overnight in 0.25% Coomassie Blue R (Sigma) dissolved in 45% methanol, 9% acetic acid, and destained in 45% methanol, 9% acetic acid. Following drying, −70°C autoradiographic exposures of the gels were made on Kodak XR-5 film using the DuPont Cronex Lightning-Plus Intensifying Screen for enhanced sensitivity. Standards used and their molecular weights were as follows: ovalbumin, 45,000 (Sigma), bovine serum albumin, 64,000 (Sigma), phosphorylase A, 92,500 (Sigma), β-galactosidase, 116,000 (P-L Biochemicals, Milwaukee), xanthine oxidase, 137,000 (Dr. Gene Nathans, University of Chicago), and RNA polymerase, 39,000, 155,000, 165,000 (Boehringer Mannheim Biochemicals).

RESULTS

ABA Derivatization of Insulin

The extent of derivatization of insulin by NHS-ABA was determined by electrophoresis of iodinated, derivatized insulin samples on pH 4.5 tube gels [16] containing 8 M urea. Derivatization was dependent upon the concentration of NHS-ABA present in the reaction mixture as shown in Figure 1. Table I provides a quantitative summary of these data and shows that with 1-mM reagent present, the predominant reaction product was monoazidobenzoyl insulin, while approximately 1/3 of the insulin remained unaltered. With 2-mM reagent, mono-derivatized insulin predominated, but about 1/4 of the insulin was di-derivatized. At 5-mM NHS-ABA, the resultant insulin mixture was composed primarily of di- and triazidobenzoyl products with about 1/4 of the insulin remaining as mono-derivative. Using 10-mM reagent, more than 90% of the reaction products were di- or triazidobenzoyl derivatives of insulin, the remainder being mono-derivatized insulin.

Monoazidobenzoyl-insulin was further analyzed to determine the preferential posi-

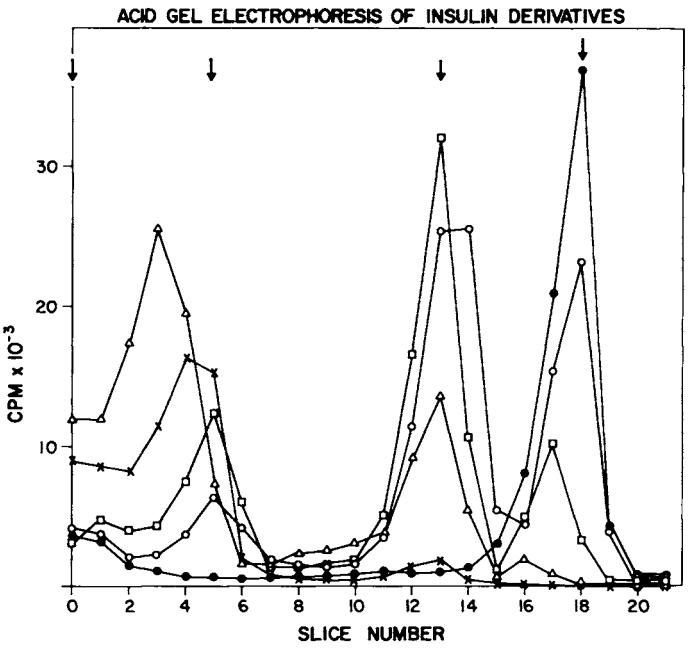

Fig. 1. Insulin derivatization with varying concentrations of NHS-ABA in the reaction mixture. The extent of derivatization was determined by electrophoresis of iodinated, azidobenzoyl-insulin samples on pH 4.5 polyacrylamide gels [16] containing 8 M urea. From left to right, arrows indicate migration position of tri-, di-, mono-, and underivatized insulin (●–● = 0 mM NHS-ABA in reaction mixture; ○–○ = 1 mM; □–□ = 2 mM; △–△ = 5 mM; X–X = 10 mM). Gels were calibrated with desamidoinsulin and with a monoderivatized insulin kindly provided by Richard Assoyan, Department of Biochemistry, University of Chicago.

TABLE I. Insulin Derivatization by Photoactivatable Reagent NHS-ABA Expressed as % of Total Products*

Reagent concentration	Un-	Mono-	Di-	Tri-
1 mM	34.0	51.7	14.3	0
2 mM	15.6	52.6	24.0	7.8
5 mM	2.5	26.3	71.1	
10 mM	0	6.7	93.3	

*Insulin was derivatized using 1, 2, 5, or 10 mM NHS-ABA as described in Experimental Procedures. The extent of insulin derivatization was evaluated by running samples on pH 4.5 polyacrylamide gels [16] containing 8 M urea. Gels were sliced and counted, and quantitation of the results was done by weighing cutout peak areas on an analytical balance.

tion of derivatization. Following oxidative sulfitolysis (3 h at 37°C in 0.2 M phosphate buffer at pH 7.5, containing 75 mM $Na_2S_4O_6$ and 270 mM Na_2SO_3 with 8 M urea) the resulting peptides were examined by paper electrophoresis in 30% formic acid. Under these conditions, only a small fraction of the A-chain exhibited altered mobility, while a major portion of the B-chain migrated more slowly. Thus, the photoactivatable reagent reacted mainly with amino groups at position B1 or B29. Numerous previous studies have indi-

cated that modifications of insulin in these positions does not drastically alter its biological activity [18].

Photoactivated Cross-Linking of ABA-^{125}I-Insulin to Albumin and Monolayer-Cultured H4 Cells

When 50 μl of 0.15 M phosphate buffer (pH 7.0) containing 12.5 mg of normal human albumin and 7 μCi of azidobenzoyl-^{125}I-insulin prepared with 1 or 10 mM NHS-ABA were exposed to ultraviolet irradiation with a UVS-11 mineralight for 30 min at room temperature, significant cross-linking was observed. Analysis of the samples in comparison with non-irradiated samples by gel chromatography on Biogel P-30 in 3 M acetic acid is shown in Table II. Clearly, photoactivation of ABA-^{125}I-insulin resulted in an increase of radiolabeled high-molecular-weight products concomitant with a decrease in the insulin peak. These results are consistent with a light-dependent covalent linking of insulin to albumin. While the proportion of high-molecular-weight light-independent aggregates was slightly increased with the more highly derivatized insulin preparation (10 mM NHS-ABA), covalent multimers of insulin were never observed.

In similar studies, direct photoaffinity labeling of H4 hepatoma cell surface receptors using ABA-^{125}I-insulin was carried out as described in Methods. Following insulin binding, the monolayers were rinsed, photoactivated, dissolved in 88% formic acid, and analyzed directly by chromatography on Bio-Gel P-30 in 3 M acetic acid. As in the previous experiment, increased formation of ABA-^{125}I-insulin-labeled high-molecular-weight complexes was observed (Fig. 2). Again, complex formation was light-dependent and presumably represented ^{125}I-insulin covalently linked to receptors.

The cross-linking efficiency of ABA-^{125}I-insulin was dependent upon the degree of azidobenzoyl derivatization of the insulin as demonstrated by the experiments summarized in Table III. H4 cells were initially labeled by a 2-h, 15°C incubation with ABA-^{125}I-insulin (1–2 μCi). Excess insulin was rinsed from the monolayer cultures, and cells were either 1) solubilized for determination of initial counts bound, 2) photoactivated and postincubated at 37°C for 10 min in fresh medium, or 3) postincubated at 37°C without photoactivation. Following postincubation, the difference between cell-bound counts in photoactivated and unactivated samples was apparently due to covalent insulin-receptor coupling. Although highly derivatized insulin preparations (5, 10 mM NHS-ABA in coupling mixture) showed remarkable cross-linking efficiency, the efficacy of the modified hormone in initial

TABLE II. P-30 Column Elution Profile for Insulin–Normal Human Albumin Mixture*

Derivatizing concentration of NHS-ABA	UVS-11 photoactivation	%Total counts		
		Void peak	Insulin peak	Column peak
1 mM	+	10.8	88.4	0.8
1 mM	–	1.3	97.7	1.0
10 mM	+	13.4	84.5	2.1
10 mM	–	3.0	94.9	2.1

*P-30 column elution profile for insulin–normal human albumin mixtures. Fifty μl of 0.15 M phosphate buffer (pH 7.0) containing 12.5 mg of normal human albumin and 7 μCi of ABA-^{125}I-insulin prepared with 1 or 10 mM reagent were exposed to ultraviolet irradiation for 30 min at room temperature and chromatographed on Biogel P-30 in 3 M acetic acid. Nonirradiated samples were run as controls. The void volume peak represented labeled high-molecular-weight products, and the column peak represented small degradation products of the labeled insulin.

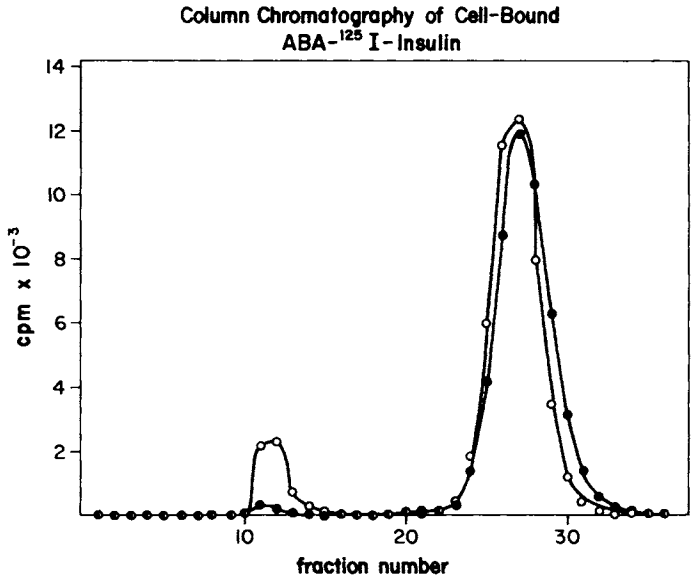

Fig. 2. Column chromatography of cell-bound ABA-^{125}I-insulin. Following insulin binding in the dark, the monolayer-cultured H4 cells were rinsed, photoactivated (•—•) or maintained dark (○—○), dissolved in 88% formic acid, and chromatographed on Bio-Gel P-30 in 3 M acetic acid. The peaks on the right represent ^{125}I-insulin, and the smaller peaks on the left represent ^{125}I-insulin associated with high-molecular-weight products, presumably receptor proteins. Clearly, photoactivation of the receptor-bound ABA-^{125}I-insulin is essential for formation of the high-molecular-weight products.

binding was markedly reduced. In contrast, minimally derivatized insulin (1 mM NHS-ABA) bound to cells more readily, but had decreased crosslinking capabilities. Combining both the binding and cross-linking properties of the various batches of azidobenzoyl-insulin by determining the percent of counts initially added that ultimately become cross-linked, the preparation giving the most efficient labeling was that which had been prepared with 1 mM NHS-ABA in the reaction mixture and consisted mainly of the monoderivative. The overall efficiency of this preparation was approximately 2, 3, and 5 times greater than that of the 2 mM, 5 mM, and 10 mM preparations, respectively. For this reason, insulin derivatized with 1 mM NHS-ABA was used for the remainder of these experiments.

Electrophoretic Analysis of the Insulin-Receptor Complex

To investigate further the nature of the products generated by photoactivation of receptor-bound ABA-^{125}I-insulin, cell samples were prepared as described in Methods and electrophoresed under reducing conditions on 7.5% polyacrylamide-SDS gels with 5.0% stacker gels according to the discontinuous system of Laemmli [17]. As shown in Figure 3A, autoradiography of dried gels indicates the light-dependent generation of 3 high-molecular-weight bands; a major band of approximate mol wt 130,000 was observed along with minor bands at 95,000, and at 40,000. A diffuse band of material at the top of the gel slab representing material of mol wt greater than 250,000 was also observed. Under non-reducing conditions, the proportion of this higher molecular weight material was markedly increased.

TABLE III. Cross-Linking Efficiency of ABA-^{125}I-Insulin to H4 Cells*

Derivatizing concentration of NHA-ABA	Photoactivation	Postincubation	Counts bound as (% of counts added)	% Bound counts remaining cell-associated	Apparent % cross-linking	% Counts added that become cross-linking
1 mM						
a	–	–	3.7%			
b	+	+		24.7%		
c	–	+		6.7%	18.0%	0.67%
2 mM						
a	–	–	2.1%			
b	+	+		23.1%		
c	–	+		9.8%	13.3%	0.28%
5 mM						
a	–	–	0.7%			
b	+	+		37.7%		
c	–	+		11.1%	26.6%	0.19%
10 mM						
a	–	–	0.5%			
b	+	+		41.8%		
c	–	+		14.8%	27.0%	0.14%

*Cross-linking efficiency of ABA-^{125}I-insulin to H4 cells. H4 cells were labeled by a 2-h, 15°C incubation with 1–2 μCi of ABA-^{125}I-insulin. Unbound insulin was rinsed from the cultures, and cells were either 1) solubilized for determination of initial counts bound, 2) photoactivated and postincubated at 37°C for 10 min in fresh medium, or 3) postincubated at 37°C without photoactivation. Following postincubation, the difference between cell-bound counts in photoactivated and unactivated samples was apparently due to covalent insulin-receptor coupling.

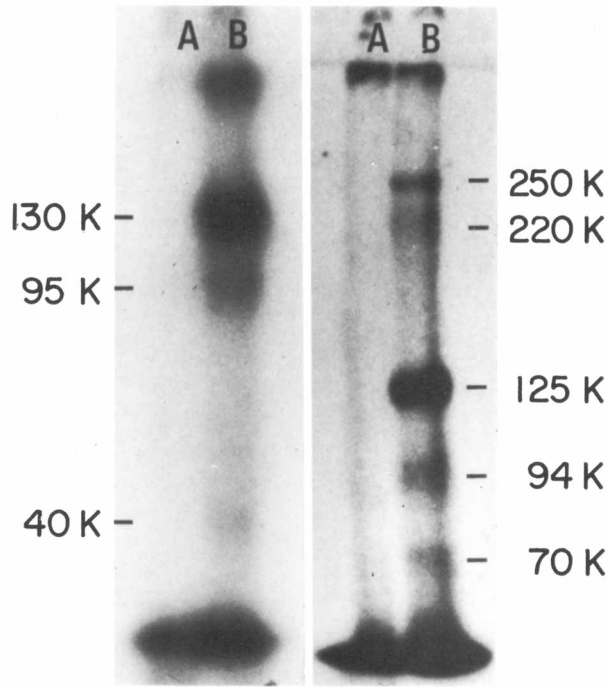

Fig. 3. SDS-polyacrylamide gel electrophoresis of labeled receptor proteins under reducing conditions. Left. Autoradiography of a dried 7.5% gel with a 5.0% stacker indicates light-dependent generation of 3 high-molecular-weight bands – a major band of apparent mol wt 130,000 and minor bands at 95,000 and 40,000. A, without photoactivation; B, with photoactivation. Right. Autoradiography of a dried 5.0% gel with a 3.0% stacker shows, from top to bottom, labeled bands at apparent molecular weights 250,000, 220,000, 125,000 predominating bands 94,000, and 72,000. A, without photoactivation; B, with photoactivation. The material at the bottom of the gel represents free insulin and/or its chains. Estimated molecular weights are indicated along the margins.

When identically labeled cell samples were run on 5.0% polyacrylamide-SDS gels with 3.0% stacker gels (Fig. 3B), the diffuse band at the top of 7.5% gels was resolved as a nonspecific component remaining at the gel top, a specific component with a mol wt of 250,000, and a diffuse minor band with an approximate mol wt of 220,000. The predominant band at approximately 125,000 and the minor band at 94,000 are both correspondent with bands previously identified on the 7.5% gels. Although the smallest receptor component (40,000 on the 7.5% gel) migrated with the tracking dye on the 5.0% gel, an additional minor component of about 70,000 was also resolved. When labeled components were further analyzed on gels of varying acrylamide concentrations in the range of 5–10%, aberrant migration of the 3 predominent labeled bands at 130k, 95k, and 40k was not observed. While altered migration due to the presence of carbohydrate might be anticipated [19], these results suggest that the proportion of receptor-associated carbohydrate is probably relatively small.

The 3 main bands (130k, 95k, 40k) become labeled in a time-dependent and parallel fashion, as shown in the autoradiogram in Figure 4. In this experiment, cells were incubated for 10, 20, 30, 60, or 120 min at 15°C with ABA-^{125}I-insulin and were rinsed, photoactivated, and solubilized and run on 7.5% Laemmli gels with 5.0% stackers [17]. Time-de-

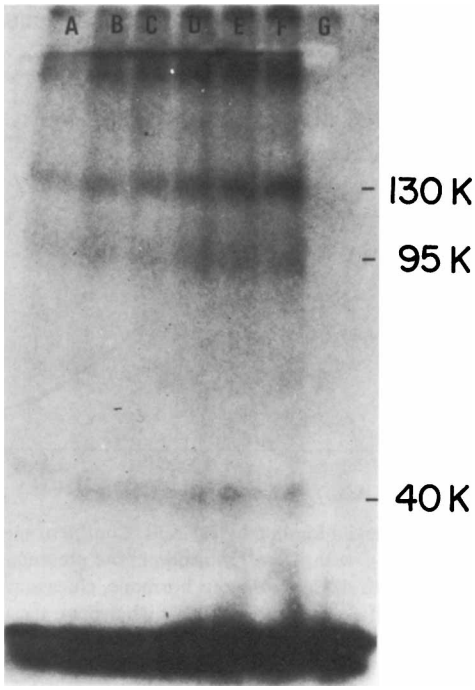

Fig. 4. Time dependency for receptor complex formation. Cells were incubated at 15°C with ABA-^{125}I-insulin for 10 (A), 20 (B), 30 (C), 60 (D), 90 (E), or 120 (F) min, and subsequently rinsed, photoactivated, solubilized, and run on a 7.5% gel with a 5.0% stacker. G represents the control condition of 120 min incubation with ABA-^{125}I-insulin, but without photoactivation. Estimated molecular weights are indicated on the right margin.

pendent formation of the labeled receptor component bands was further substantiated by direct counting of bands cut from the dried slab gels. When binding of ABA-^{125}I-insulin was carried out at 37°C, the same bands were labeled, although binding occurred more rapidly, as expected.

Furthermore, the binding of ABA-^{125}I-insulin to H4 hepatoma cells was specific, as was the light-dependent generation of the radioactively labeled 130k, 95k, and 40k bands. Figure 5 shows the progressive displacement of ABA-^{125}I-insulin binding by increasing concentrations of native insulin and demonstrates that glucagon did not inhibit binding. Autoradiograms prepared from slab gel analysis of similar experimental samples demonstrated that all of the photolabeled bands were displaced equally well by native hormone (data not shown). The slight decrease in K_D for this preparation (Fig. 5) in comparison to the value previously observed for native insulin [12] indicates that the monoderivatized material has about 1/3–1/2 the affinity of native insulin for the receptor. For this reason it was not considered necessary to purify this preparation further.

To determine whether insulin receptors were processed by the cells as previously reported for epidermal growth factor [20], receptors were initially covalently labeled as described in Methods and subsequently postincubated at 37°C in fresh medium for periods up to 3 h. Under these conditions, we failed to observe the generation of any radiolabeled receptor breakdown products as shown in Figure 6, even though control experiments (not

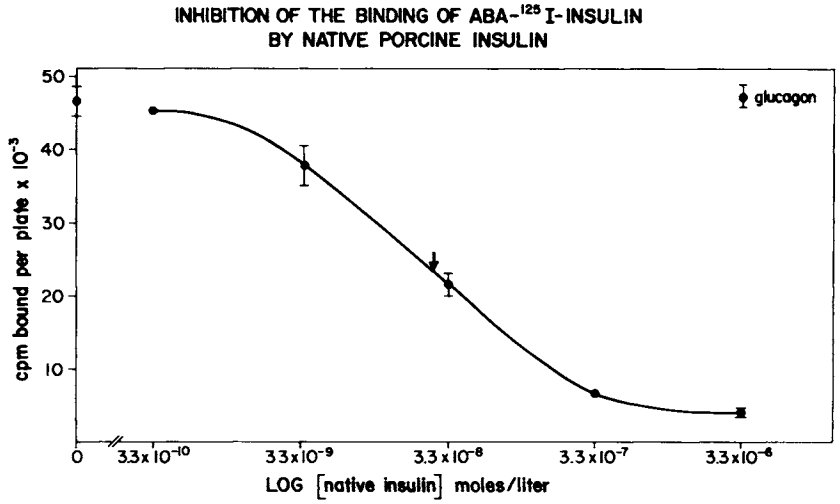

Fig. 5. Specificity of ABA-^{125}I-insulin binding by H4 cells. Confluent monolayer cultures of H4 cells were incubated for 30 min at 30°C with ABA-^{125}I-insulin in the presence of varying concentrations of native insulin, as indicated, or with the heterologous hormone, glucagon. The K_D value of 26 nM for ABA-^{125}I-insulin (arrow) was similar to, though slightly higher than, the value of 14 nM obtained from studies with ^{125}I-insulin [23].

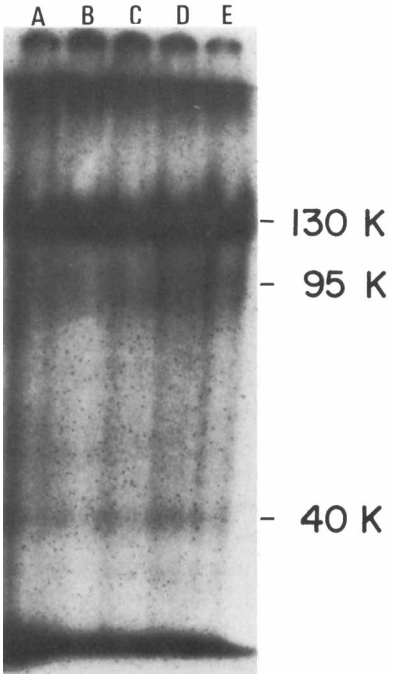

Fig. 6. Cellular receptor processing experiment. Cell-bound ABA-^{125}I-insulin (120 min, 15°C incubation) was covalently linked to receptor components by photoactivation, and cells were subsequently postincubated at 37°C for various intervals: A = 0 min; B = 5 min; C = 10 min; D = 20 min; E = 60 min. Following rinsing and solubilization, labeled components were analyzed on 7.5% gels with 5.0% stackers. Bars indicate the 3 receptor-associated protein bands (130k, 95k, 40k), which do not appear to change in intensity with time.

shown) showed nearly normal processing of insulin by cells exposed to ultraviolet irradiation. When labeled receptor component bands were analyzed in 3 separate experiments, there did not appear to be any substantial loss of radioactivity during the course of post-incubation. Also, under our experimental conditions, we did not detect any covalent cross-linking of native hormone and receptor as reported for thrombin [21] and epidermal growth factor [22], or as suggested recently for α_2-macroglobulin [23].

DISCUSSION

Using a photoreactive, iodinated derivative of insulin (ABA-^{125}I-insulin) to specifically label cell surface components of the insulin receptor complex in insulin-responsive hepatoma cells [24], a predominant band of apparent* molecular weight 130,000 was observed by SDS polyacrylamide gel electrophoresis. This major receptor component is likely to be the same component as was identified in previous investigations using rat liver and adipocyte plasma membranes [25–31], as well as human placental membranes [26]. Variability in the molecular weight reported for this major receptor component from 125,000 to 135,000 presumably has resulted from the use of different protein standards or systems. Minor labeled components of the H4 cell insulin-receptor complex with molecular weights of 95,000 and 40,000 were also observed. Yip et al [30] have previously reported the labeling of a 90,000 mol wt band in liver plasma membranes by azidobenzoyl-insulin derivatives, while Jacobs et al [26] have observed the presence of a similar band as a minor component in the preparation of purified rat liver insulin receptors, along with other minor bands at 75,000 and 45,000. Iodination of these purified insulin receptors resulted in the labeling of major bands of apparent mol wt 135,000 and 45,000 on SDS gels [26]. These bands probably correspond to the photolabeled bands at 130,000 and 40,000 described here. Thus, while similar receptor subunit bands have been observed previously [26–30], they have only been simultaneously present in early stages of preparative receptor isolation [26]. The fact that all these components become labeled when intact, insulin-responsive cells are studied implies that they are all contiguous and externally oriented in the unperturbed membrane. Indeed, previous studies on insulin receptors in isolated plasma membranes have shown that the molecular weight of the intact receptor complex is about 300,000 [26, 27, 32].

The photolabeled bands observed here (130k, 95k, and 40k) were insulin-specific, since their labeling could be prevented by simultaneous incubation with native insulin. Also, association of the radioactive, photoactivatable insulin derivative with these receptor-related bands was both time- and light-dependent. We can therefore tentatively conclude that the major protein band at 130,000 may represent the main insulin-binding subunit of the receptor and that the minor protein bands at 95,000 and 40,000 represent components of the receptor complex or other receptor-associated proteins. The smaller components may indeed play important structural roles as, for example, in orientating the receptor complex within the membrane, or alternatively, they may be important in mediating, either directly or indirectly, the biological effects of insulin [1–3].

It seems highly unlikely that the 40,000 and 95,000 receptor components simply represent degradative fragments of the main 130,000 component, since they were observed in experiments where binding occurred at 15°C, a temperature which prevents uptake and

*The actual molecular weight of these components would presumably be roughly 3,000 less than observed due to the weight of the associated photoligand (derivatized insulin B chain).

degradation of insulin and, presumably, of the receptor as well [7, 8]. Also, when the receptor was insulin-labeled at 15°C, photoactivated for hormone-receptor cross-linking, and postincubated at 37°C for intervals up to 3 h, the smaller subunits (95k, 40k) failed to appear in increased proportions. Furthermore, we did not detect the appearance of any labeled breakdown products of the covalently linked insulin-receptor complex as previously observed for epidermal growth factor [20], although it remains possible that the modified receptor does not behave normally in terms of its uptake and intracellular distribution. However, no substantial loss of radioactivity from the labeled bands could be observed over periods up to 3 h. Our failure to observe receptor breakdown is consistent with observations in our laboratory that H4 cells fail to show significant receptor down regulation even in the presence of 1 μg/ml insulin for 24 h [Miller, Hofmann, and Steiner; unpublished results]. The results are thus consistent with proposals that intact receptors may be recycled to the plasma membrane [33, 34] or that modification of insulin receptors or of essential cellular components by cross-linking and/or irradiation may alter their behavior.

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