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Insulin binding to solubilized material from fat cell membranes: Evidence for two binding species

(insulin receptor/polyacrylamide gel electrophoresis/adipocyte)

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ABSTRACT **The components of fat cell membranes responsible for the binding of insulin were solubilized by treatment with the nonionic detergent Triton X-100. By using a polyethylene glycol precipitation method to assay specific insulin binding, the soluble preparation was shown to have insulin-binding characteristics similar to those of intact fat cells. Further studies of this preparation by polyacrylamide gel electrophoresis in the presence of ^{125}I-labeled insulin demonstrated two distinct insulin binding activities, designated species I and II. The two species were separated by electrophoresis in the absence of iodo-labeled hormone and eluted from the gel. Scatchard analysis of the insulin binding data for species I showed a curvilinear plot with the initial portion having a K_d of 1.3×10^{-10} M. The Scatchard plot for species II was linear with a K_d of 6.0×10^{-9} M. Desoctapeptide insulin and glucagon failed to compete for the insulin-binding sites in both species whereas desalanine insulin was an effective competitor. High concentrations of proinsulin competed with the iodo-labeled hormone for binding to species I but not to species II. In the presence of a low concentration of ^{125}I-labeled insulin (0.3 nM) some species I activity appeared to be converted to species II activity; there was no evidence of interconversion between the two species in the absence of insulin. Neither species degraded insulin as measured by trichloroacetic acid precipitation or rebinding to intact fat cells. These findings indicate the existence in the adipocyte plasma membrane of two insulin-binding species that have distinct physicochemical properties.**

The binding of insulin to target cells has been extensively studied in many laboratories (1–5). Evidence from these studies suggests that this interaction is a complex process and not a simple bimolecular association of the hormone with one class of binding sites. This nonclassical behavior is indicated primarily by the curvilinear Scatchard plot (6) obtained from analysis of insulin-binding data and the accelerated dissociation of bound ^{125}I-labeled insulin (^{125}I-insulin) after the addition of native hormone (7).

Several models have been proposed to explain these findings. Initially, the curvilinear Scatchard plot was attributed to the presence of two or more classes of binding sites that have different affinities for insulin (8). However, this model alone cannot accommodate the results of the dissociation studies. More recently, DeMeyts and coworkers (7, 9), who first described the effect of native hormone on the dissociation of labeled insulin, proposed a negative cooperative model that involves insulin binding to a homogeneous class of empty high-affinity sites (10). These sites then undergo conformational changes through site–site interactions that result in their transformation to a low-affinity state. A third model, the mobile receptor hypothesis, has been advanced by Jacobs and Cuatrecasas (11), by Boeynaems and Dumont (12), and by De Häen (13); it is based on the possible existence in the membrane of an equilibrium between receptors and receptor–effector complexes that differ in their affinities for insulin. Both the negative cooperativity model and the mobile receptor hypothesis provide explanations for the shape of the Scatchard plots and for the dissociation data.

Support for the validity of these or other possible models requires more detailed information on the insulin binding structure(s) than is now available. In the present report, we show evidence that two distinct insulin-binding components exist in detergent-solubilized material prepared from membranes of adipocytes, a well-established target cell for insulin. The two species have significantly different insulin-binding characteristics, which may explain some of the features of the insulin–adipocyte interaction.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats weighing 175–210 g were obtained from Charles River Laboratories. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N'N'*-tetramethylethylenediamine, chloramine-T, and sodium metabisulfite were purchased from Eastman. Triton X-100 was purchased from J. T. Baker Company. Sodium iodide-125 was obtained from New England Nuclear. Porcine insulin, desalanine insulin, desoctapeptide insulin, proinsulin, and glucagon were gifts of the Eli Lilly Co.

Preparation of Soluble Insulin-Binding Material. Isolated fat cells were prepared from rat epididymal fat pads as described (14). Purified plasma membranes were prepared from the adipocytes as described by Czech and Lynn (15). Soluble insulin-binding components were prepared by treatment of the plasma membranes (5–10 mg of protein per ml) with 1% (vol/vol) Triton X-100 for 30 min at 21° (16). Insoluble material was removed by centrifugation at 150,000 × *g* for 90 min at 4°.

Assay of ^{125}I-Insulin Binding to Solubilized Binding Components. ^{125}I-Insulin (100–200 Ci/g) was prepared by the chloramine-T method (16). Specific binding of ^{125}I-insulin by the soluble material was measured by using the polyethylene glycol precipitation method (16) except that the incubation was conducted for 16 hr at 4°.

Gel Electrophoresis of Soluble Insulin-Binding Material. Five percent polyacrylamide gels (12.5 cm long) and Tris/glycine electrode buffer were prepared as described by Davis (17) and modified by the addition of Triton X-100 (final concentration, 0.1%, vol/vol).

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Abbreviation: ^{125}I-insulin, ^{125}I-labeled insulin.

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Binding material (100 μg of protein) was incubated with labeled insulin for 16 hr at 4° in the presence or absence of excess native insulin. One hundred microliters of this mixture containing 7 mg of sucrose was layered on the gel. Electrophoresis was carried out for 2.5 hr at 4° with a current of 2 mA per gel. The gels were then removed from the tubes and treated for 1 hr with 12.5% trichloroacetic acid. The gels were sliced into 2-mm segments and the radioactivity was determined with a Searle model 1097 gamma counter.

Insulin-binding activity was also eluted from gel segments after electrophoresis of the detergent-soluble preparation. In these experiments, 100 μl of the soluble preparation was electrophoresed as described above except that labeled and unlabeled insulin were absent. The gel was then sliced at 4° and segments consisting of six 2-mm slices were placed in tubes that contained 1 ml of 50 mM Tris·HCl, pH 7.6/0.1% (wt/vol) albumin/0.1% (vol/vol) Triton X-100. The elution was continued for 16 hr in the cold and the eluates were assayed for soluble insulin-binding activity or used in further electrophoresis studies after concentration in an ultrafiltration cell (UM20E membrane).

Degradation of ^{125}I-Insulin. Degradation of ^{125}I-insulin was estimated by the trichloroacetic acid precipitation method (18) and by rebinding studies using isolated fat cells (18).

Protein Determination. Protein was measured by the method of Miller (19) with bovine serum albumin standards.

RESULTS

Studies of the Soluble Insulin-Binding Preparation. In agreement with previous work (16), the Triton X-100-solubilized material specifically bound ^{125}I-insulin as demonstrated by competition studies with insulin and insulin analogues (data not shown). Scatchard analysis (6) of the results from insulin-binding studies with the soluble preparation is shown in Fig. 1. As demonstrated with various tissues including fat cells, the Scatchard plot is nonlinear with an upward concavity. The high-affinity portion of the curve has an estimated K_d of 1–2 $\times 10^{-10}$ M which is similar to the values reported for insulin binding to membranes of insulin target tissues (20).

The soluble preparation degraded a small percentage of the labeled hormone. As measured by trichloroacetic acid precipitation, only 2% of the ^{125}I-insulin (0.3 nM) was degraded after incubation for 1 hr at 37° and no degradation was found when the incubation conditions were 4° for 16 hr or 24° for 1 hr (data not shown).

Gel Electrophoresis of the Insulin-Binding Preparation with ^{125}I-Insulin. Gel electrophoresis was conducted on the soluble insulin-binding preparation after a 16-hr incubation at 4° with ^{125}I-insulin in the presence or absence of excess native insulin (Fig. 2). After electrophoresis in the absence of native insulin, two major peaks of radioactivity were apparent in the areas of gel slices 10 (peak I) and 40 (Fig. 2*A*). In the presence of excess native insulin (Fig. 2*B*), the amounts of radioactivity were decreased in the area of gel slice 10 and the trailing shoulder (peak II) of the large peak. Fig. 2*C* indicates the location of the free (unbound) ^{125}I-insulin which runs in the area of gel slice 40 and constitutes the large peak of radioactivity. In these experiments, essentially all of the radioactivity placed on the gels was recovered.

Elution of the Insulin-Binding Species from the Polyacrylamide Gel. Insulin-binding activity was also eluted from the gels (Fig. 3). In this experiment, the solubilized preparation was placed on the gel in the absence of either labeled or unlabeled insulin. After the electrophoresis, the gels were sliced and eluted and the amount of insulin-binding activity was determined by the soluble binding assay. Gel slices 7–12 contained the major portion of peak I activity; peak II activity was present in the eluate from gel slices 25–30. Eluates from the other segments did not contain significant insulin-binding activity except for the small amounts present at the end of the gel.

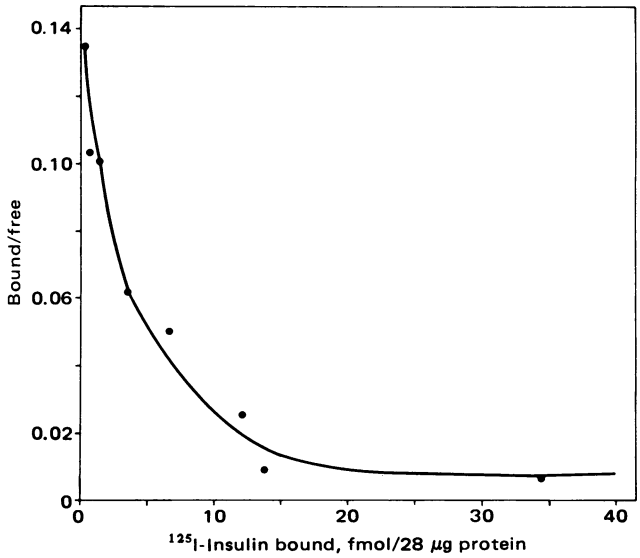

FIG. 1. Scatchard plot of the specific binding of ^{125}I-insulin to solubilized material from fat cell plasma membrane. The ^{125}I-insulin concentrations ranged from 30 pM to 10 nM, and the protein concentration of the binding material sample was 56 μg/ml. The data are from a representative experiment that was repeated six times. The high-affinity K_d calculated from these experiments ranged from 1 to 5×10^{-10} M.

Table 1 gives an indication of the specificity for the association between insulin and the two insulin-binding species. Desalanine insulin, which is as biologically active as the native hormone (21), was almost as potent as insulin in displacing ^{125}I-insulin from peak I and competed as effectively as insulin for the binding to the peak II species. Desoctapeptide insulin, which has almost no biological activity (20), and glucagon failed to compete with ^{125}I-insulin for binding to either peak. The major difference in the specificity for hormone binding between the two binding species was evident in the proinsulin competition studies. Although not as effective as insulin in displacing the labeled hormone from peak I, a high concentration of proinsulin inhibited a major portion of the specific insulin binding. Proinsulin did not compete with ^{125}I-insulin for binding to peak II.

Table 1. Specificity of the insulin-binding species

Additions	% inhibition of specific insulin-binding activity*†	
	Peak I	Peak II
Insulin, 100 ng/ml‡	100	100
Desalanine insulin, 100 ng/ml	65–100	100
Desoctapeptide insulin, 500 ng/ml	NID–10	NID
Proinsulin,		
500 ng/ml	60–81	NID
100 ng/ml	21–40	NID
Glucagon, 1 μg/ml	NID	NID

The data are the ranges of three separate experiments.
* Concentration of ^{125}I-insulin was 0.3 nM.
† NID, no inhibition detected.
‡ This concentration yields 95–100% displacement of ^{125}I-insulin binding to soluble binding material.

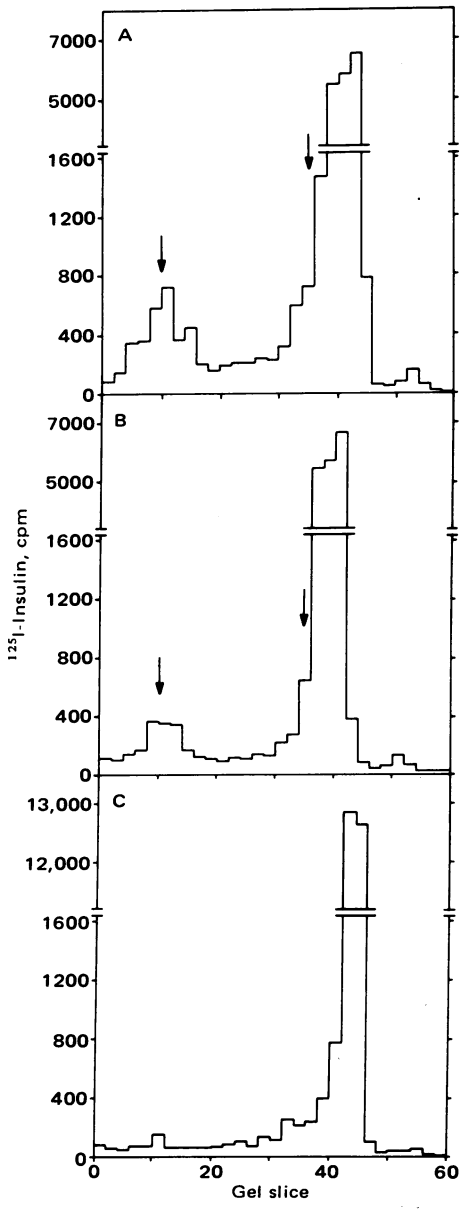

FIG. 2. Gel electrophoresis of the soluble binding material in the presence of ^{125}I-insulin. The soluble preparation was incubated for 16 hr at 4° with 0.3 nM labeled insulin both in the absence (*A*) and presence (*B*) of unlabeled insulin (0.7 μM). The resultant mixtures were subjected to electrophoresis and the radioactivity of the gel slices was determined (direction of migration, 1 → 60; cathode in upper chamber). (*C*) Position of ^{125}I-insulin. Arrows, presence of specific insulin-binding activity. An estimate of specific insulin binding in a segment of gel can be obtained by subtracting the radioactivity shown in *B* (nonspecific binding plus some free ^{125}I-insulin) from that in *A* (total binding plus free ^{125}I-insulin). In gel slices 8–12 (R_F = 0.20) and in slices 34 (R_F = 0.54) through 38, the amount of specific binding was 744 and 1671 cpm, respectively. The data are from a representative experiment repeated 10 separate times.

Fig. 4 shows a Scatchard analysis of the ^{125}I-insulin binding data for peaks I and II. The analysis for peak I gave a curvilinear plot with an apparent K_d of 1.3×10^{-10} M for the high-affinity component. Insulin binding to the peak II species differed from that of peak I; the plot is linear and the affinity for the iodo-labeled hormone is much lower ($K_d = 6 \times 10^{-9}$ M).

In addition to the insulin-binding studies, the possibility that one or both species degraded insulin was investigated by incubating the eluates with the labeled hormone for 1 hr at 24°

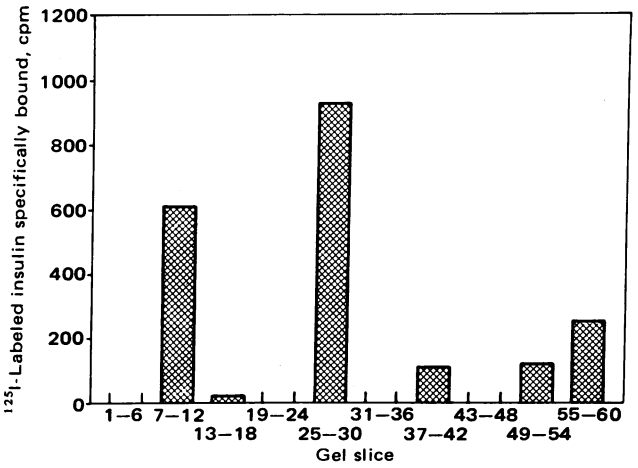

FIG. 3. Elution of insulin-binding activity from gels after electrophoresis. The eluates were assayed for specific binding by the polyethylene glycol method. The radioactivity in slices 7–12 corresponds to peak I in Fig. 2; and peak II activity is present in slices 25–30. The data are from a representative experiment repeated six separate times.

or 37°. However, under these conditions and using two different methods for detection (trichloroacetic acid precipitation and rebinding studies to fat cells) we could not detect any degradation caused by either binding species (data not shown).

Electrophoresis of the Isolated Insulin-Binding Species. In an effort to determine if there is any interconversion of the binding species (peaks I and II), the eluates containing insulin-binding activity were subjected to gel electrophoresis a second time after an incubation with ^{125}I-insulin. Fig. 5 shows the results from the electrophoresis of peak I and the labeled hormone. Two peaks of specific radioactivity are indicated by the arrows: a large peak exists at gel slice 10 (the position of peak I) and a smaller peak (peak II) that trailed the large free ^{125}I-labeled peak (gel slices 32–40). In the same type of experiment, we found no evidence that any of peak II activity was converted to peak I (data not shown).

The binding species were also subjected to electrophoresis in the absence of labeled or native hormone, and the position of the binding activities was determined by the soluble receptor

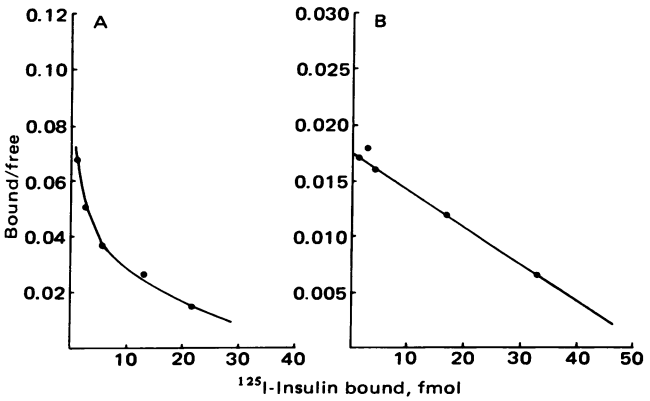

FIG. 4. Scatchard analysis of the insulin-binding activity for peaks I and II eluted from polyacrylamide gels. (*A*) Results for peak I (slices 7–12). (*B*) Peak II activity (slices 25–30). Scatchard analysis of peak I activity shows a curvilinear profile whose high-affinity segment yields a K_d of 1.3×10^{-10} M. The profile for peak II is a linear plot and has a K_d of 6.0×10^{-9} M. The results are the mean of four separate experiments.

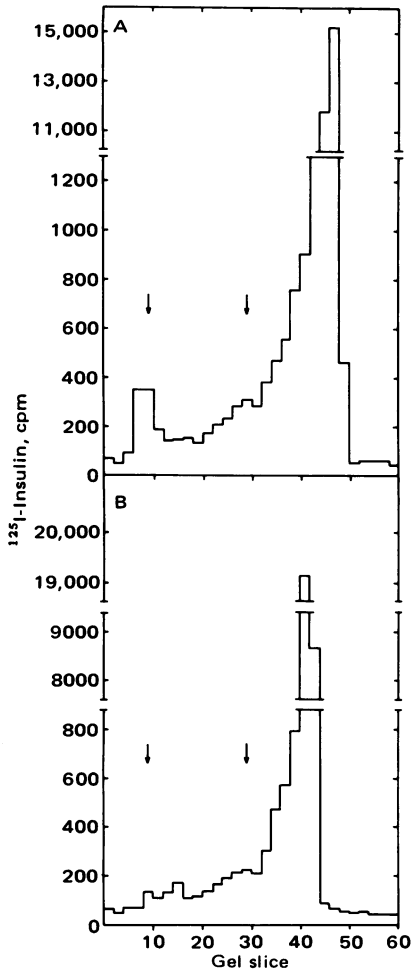

FIG. 5. Electrophoresis of peak I activity in the presence of ^{125}I-insulin. *A* and *B* correspond to those shown in Fig. 2 except that isolated peak I activity replaces the soluble binding material. Arrows, the presence of specific insulin-binding activity (approximately 492 cpm for slices 6–10 and 155 cpm for slices 26–30). The experiment used ^{125}I-insulin at 0.3 nM. The results are from a representative experiment repeated four separate times.

assay after elution of the gel segments. Only one peak of binding activity, which migrated as peak I, was detected after electrophoresis of peak I. Similarly, electrophoresis of peak II resulted in the binding activity migrating only as peak II (data not shown).

DISCUSSION

Studies of insulin-binding sites have generally been carried out with intact cells or purified membrane preparations. This approach, however, does not readily lend itself to characterizing the physicochemical features of the binding species. Recently, insulin-binding material has been solubilized from target cell membranes and shown to retain many (perhaps all) of the insulin binding characteristics of the parent tissue (10, 16, 22, 23). It is therefore possible to gain information about the membrane components responsible for insulin binding by applying conventional methods used in studies of protein chemistry.

In the present study, electrophoresis of the detergent-soluble material indicates the existence of at least two insulin-binding species in the fat cell membrane. Their presence was shown in two ways: (*i*) by locating areas of specific binding activity on the gels after electrophoresis in the presence of labeled insulin and (*ii*) by electrophoresis in the absence of insulin and subsequent elution from the gel of insulin-binding activity. The second method has the advantages of allowing the examination of the entire gel (e.g., the large free ^{125}I-insulin peak obscures a portion of the gel including part of the peak II binding activity) and of measuring specific insulin binding under conditions normally used in studies of the soluble insulin receptor.

The elution method also allows an examination of the insulin binding characteristics of the two species (peak I and peak II). Scatchard analysis demonstrates a functional difference between the two species; peak I appears to be responsible for high-affinity insulin-binding activity, whereas the affinity exhibited by peak II is much lower and thus may contribute to the binding found with high insulin concentrations. Another difference is the diminished effectiveness of proinsulin to compete with ^{125}I-insulin for binding to peak II.

An interesting feature which constitutes a major difference between the two species is the curvilinear Scatchard plot found in insulin binding studies with peak I. This finding is consistent with the possibility that the two binding species are related and that insulin modulates the formation of the "low" affinity species (peak II) from the high-affinity species (peak I). An initial attempt was made to test this possibility by incubating ^{125}I-insulin with peak I and then performing electrophoresis. As shown in Fig. 5, a moderate amount of specific insulin-binding activity appeared in the position of peak II that was not present before incubation with the labeled hormone. In contrast, however, when the same experiment was done using peak II activity, no apparent binding activity was detected in the position for peak I (data not shown).

In a recent study of solubilized material from turkey erythrocytes, Ginsberg *et al.* (10) detected only one insulin binding species by Sepharose 6B gel chromatography in the absence of insulin. Insulin treatment of this peak caused the appearance of a second peak of binding activity with lower molecular weight when the material was rechromatographed. Under suitable conditions, the second peak could be shown to repolymerize and form the high-molecular weight binding species. Unfortunately, no studies of the insulin binding characteristics were described for the smaller species (presumably low-affinity).

Our findings differ in certain respects from those described above. Two distinct insulin-binding species are present in the soluble material from fat cells in the absence of any treatment with insulin. Second, we have not detected any conversion of peak II material to peak I, although other incubation conditions must be tried before this is conclusively established. It is also apparent from the Scatchard plot of peak II activity that little if any peak I material was re-formed because there was no indication of a high-affinity binding process. The differences noted between these studies may be related to the different cell types used to prepare the soluble binding sites.

The present studies represent an attempt at further characterization of the insulin-binding species from a well-established target cell for insulin. The data are not sufficient at this point to argue strongly for any one of the several theories regarding the nature of the insulin-binding sites in target cells that cause the curvilinearity of the Scatchard plot (8, 9, 11–13, 24–26). The results do support multiple classes of binding sites with different affinities. However, because there is some suggestion of an insulin-mediated conversion of peak I to peak II, the two binding species may be related in some manner. This finding in itself does not constitute evidence for negative cooperativity; the model as postulated by DeMeyts *et al.* (9) would require the reconversion of some peak II activity to peak I, for which we as yet have no evidence. Other possibilities should also be

considered. For example, the association of insulin with species I could induce an irreversible dissociation of subunits or the formation of fragments that have a decreased affinity for insulin. Such a process might be related to the poorly understood phenomenon of receptor "degradation" (27). Also, it could explain why the high-affinity insulin binding sites are absent and the low-affinity sites remain on monocytes taken from patients with congenital lipodystrophy who have very high plasma insulin levels (28). Another possibility is that proteolytic cleavage of species I causes the formation of the second insulin binding peak. This possibility is unlikely, however, because solubilization of the membrane in the presence of proteolytic inhibitors (bacitracin, *N*-ethylmaleimide, Trasylol, ovomucoid trypsin inhibitor, phenylmethylsulfonyl fluoride) did not eliminate either insulin binding species (unpublished data).

It is also possible that one of the binding species represents the insulin receptor and the other species represents an insulin receptor–effector complex (11–13). The effector could be a component of the glucose transport system because in erythrocyte membranes the transport system is solubilized by the same detergent treatment used to solubilize the insulin-binding species from adipocytes (29). Finally, the formation of species II from species I after the binding of insulin may represent the second step in the sequence of events that leads to an effect of the hormone on a target cell. The presence of species II in fat cell membranes may therefore reflect *in vivo* exposure of the adipocytes to insulin. Resolution of these questions will require further characterization of the insulin-binding species and studies of their possible role in the action of insulin.

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