The Nuclear Envelope

THE MAJOR SITE OF INSULIN BINDING IN RAT LIVER NUCLEI*

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Prior studies have demonstrated the presence of specific binding sites for insulin on nuclei isolated from rat liver. In the present study to determine the subnuclear location of these insulin binding sites, intact nuclei were first incubated with 125I-insulin and then subfractionated. Approximately 70% of specific insulin binding was associated with the nuclear membrane fractions. When highly purified nuclear membranes were prepared and incubated directly with 125I-insulin, specific binding sites were demonstrable. When compared on the basis of protein content, nuclear membranes specifically bound 10 to 15 times more labeled insulin than did intact nuclei. The binding of insulin to nuclear membranes was time-dependent and saturable; Scatchard plots revealed a high affinity binding site with a Kd of 6.1 ± 0.1 nM and a lower affinity binding site with a Kd of 65.6 ± 11.8 nM. As has been reported for insulin binding sites on plasma membranes, proinsulin and desoctapeptide insulin competed for insulin binding sites on nuclear membranes in proportion to their reduced biological activities; unrelated hormones were without effect. In contrast to insulin binding sites on plasma membranes, insulin binding sites on nuclear membranes displayed neither negative cooperativity nor an enhancement of binding activity when incubated with 200 μM NaCl. The pH optimum for binding of insulin to nuclear membranes was a plateau between 6.5 and 7.25; in comparison, the pH optimum for binding to plasma membranes was a sharp peak at 8.0. These studies indicate that (a) the nuclear envelope is the major site of insulin binding to the cell nucleus; (b) the binding sites for insulin on the nuclear envelope have high affinities and are hormone specific; and (c) these binding sites have characteristics differing from those on the plasma membrane.

Studies from this laboratory indicate that insulin can enter the intact human cultured lymphocyte and bind to the nucleus (1). Further, purified nuclei isolated from both rat liver and human cultured lymphocytes contain specific binding sites for insulin (2, 3). The subnuclear locations of these insulin binding sites, however, have not been elucidated. Other experiments indicate that insulin does not bind directly to DNA, histone, and nonhistone proteins, suggesting that these nuclear binding sites for insulin are not located on chromatin. In the present study, we present evidence indicating that the majority of these nuclear binding sites for insulin are located on the nuclear envelope.

MATERIALS AND METHODS

Materials—Pork insulin (25.6 units/mg) was purchased from Elanco Products Co., Indianapolis, Ind.; other hormones were obtained as previously published (2). 125I-Insulin (80 to 120 μCi/μg) and 125I-glucagon (80 to 100 μCi/μg) were prepared as previously described (2, 4). The buffer employed in most binding studies contained 20 mM Tris/HCl, 10 mM MgCl2, 2 mM EDTA, 0.25 M sucrose, and 0.5% bovine serum albumin, w/v (pH 7.5) (binding buffer). In studies of both negative cooperativity and the influence of NaCl, a buffer containing 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 0.5% bovine serum albumin (pH 7.5) was used (see below).

Subfractionation of Nuclei after Insulin Binding—Highly purified rat liver nuclei were prepared from female Sprague-Dawley rats weighing 180 to 220 g by the method of Blobel and Potter (5). Electron micrographs were routinely obtained to ensure that there was no contamination of the preparations with other cellular organelles. When these nuclei were resuspended and centrifuged an additional time through 2.2 M sucrose, there was no change in their ability to bind insulin. This finding also indicated that the nuclei were not contaminated with other cellular fractions.

Nuclei then were incubated at 2.0 to 2.2 mg of protein/ml with 6.0 to 6.5 ng/ml of 125I-insulin (in the presence and absence of 200 μg/ml of unlabeled insulin) in binding buffer for 2 h at 23°C. Bound and free insulin were separated by centrifugation at 2,800 × g for 10 min at 4°C. The nuclear pellet was washed twice at 4°C with buffer containing 50 mM Tris/HCl (pH 7.5), 12.5 mM NaCl, 12.5 mM KCl, and 5 mM MgCl2 (Buffer A). Nuclei were then fractionated according to the procedure of Tata and Baker (6). The washed nuclei were suspended in Buffer A, kept cold in an ice bath, and exposed to a 15-s single burst of sonication (Biosonic IV sonifier at setting 50-high). Microscopic examinations were performed to ensure that over 90% of the nuclei were disrupted. The nuclear sap was separated by centrifugation (1,000 × g for 45 min at 4°C), and the pellet was suspended in...
Buffer A containing 90 mM sodium citrate and 90 mM potassium citrate (Buffer B). This suspension was placed on a discontinuous gradient with layers of 2.0, 1.72, 1.37, 1.17, and 0.88 M sucrose in Buffer B. The mixture was centrifuged at 94,000 x g for 2 h at 4°C, and the nuclear fractions were collected. With this procedure, euchromatin floats on the surface of this sucrose gradient, the nuclear membranes layer out at the 0.00/0.88 M and 0.88/1.17 M interfaces, the nucleoli are at the 1.37/1.72 M sucrose interface, and heterochromatin is found in the pellet at the bottom of the tube (6). The small amounts of material at interfaces 1.17/1.37 M and 1.72/2.0 M, which have not been completely characterized, as well as radioactivity in the nuclear sap are indicated in Fig. 1 as “other.” All the nuclear fractions then were collected and the radioactivity was measured in a well-type scintillation counter. Recovery of radioactivity was approximately 90 to 95% of total.

Preparation of Nuclear and Plasma Membranes - Purified nuclear membranes were prepared according to the procedure of Kashnig and Kasper (7, 8). Rat liver nuclei first were disrupted by sonication (Biosonik IV sonifier at setting 50-high), and the nuclear sap then was solubilized by adding potassium citrate at a final concentration of 10% w/w. To separate partially purified nuclear membranes from chromatin, the sonicated citrate-treated extract was layered over a cushion of sucrose having a density of 1.22 g/ml and containing 50 mM Tris/HCl (pH 7.5), 25 mM KCl, 5 mM MgCl₂, and 10% (w/v) potassium citrate (Buffer C), and centrifuged at 100,000 x g for 45 min at 4°C. With this procedure, partially purified nuclear membranes layer out on the top of the sucrose cushion and a chromatin-rich fraction is recovered in the pellet at the bottom of the tube. The partially purified nuclear membranes then were suspended in 7 ml of Buffer C (density 1.22 g/ml) and overlaid, in a stepwise manner, with 3.5 ml each of Buffer C solutions with densities of 1.18, 1.18, and 1.16 g/ml. This discontinuous gradient was centrifuged at 104,000 x g for 14 h at 3°C. Purified nuclear membranes were isolated at the interfaces of 1.16/1.18 and 1.18/1.20 layers.

Plasma membranes were prepared by the method of Ray (9).

The direct binding of ¹²⁵I-insulin to various cellular fractions at 23°C was performed as previously described (2); samples were incubated with ¹²⁵I-insulin plus an excess of unlabeled insulin (100 or 200 μg/ml) to correct for nonspecific binding. After separation of bound and free hormone (2), nonspecific binding (binding in the presence of 200 μg/ml of unlabeled insulin) was subtracted.

RESULTS

Binding of Insulin to Purified Nuclei Followed by Subfractionation - When purified nuclei were first incubated with ¹²⁵I-insulin and then subfractionated, nearly 70% of specific binding was found in the fractions containing the nuclear membranes (Fig. 1). Much smaller amounts of specific binding were noted in the chromatin, nucleoli, and in other nuclear fractions. This type of experiment suggested, therefore, that the nuclear membrane is the major site of insulin binding in the nucleus.

Direct Binding of Insulin to Purified Nuclear Membranes - Further evidence for this location of the insulin binding sites was obtained by directly incubating ¹²⁵I-insulin with both semipurified and purified nuclear membranes. When compared to intact nuclei (on the basis of protein content), semipurified nuclear membranes bound 3 to 4 times more of the labeled insulin, and purified nuclear membranes 10 to 15 times more, than did intact nuclei (Table D). In contrast, the chromatin fraction bound insulin less well than intact nuclei (Table I).

At 23°C, one-half maximal binding of insulin to purified nuclear membranes occurred within 20 to 40 min and maximal binding was seen within 120 min (Fig. 2). A binding plateau was maintained for 150 min or longer. Degradation of labeled insulin, as determined by the ability of the free insulin to precipitate in 10% trichloroacetic acid (10), was minimal (less than 5% of total) after 3 h of incubation (Fig. 2).

The binding of ¹²⁵I-insulin was inhibited by unlabeled insulin at concentrations as low as 1 ng/ml (160 pm); one-half

![Subnuclear localization of insulin bound to intact nuclei](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Table I</th>
<th>Binding of insulin by serial fractions in purification of rat liver nuclear membranes</th>
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<tbody>
<tr>
<td></td>
<td><strong>Bound/free</strong></td>
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<tr>
<td><strong>Nuclear fraction</strong></td>
<td></td>
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<tr>
<td>Intact nuclei</td>
<td>0.015 ± 0.003</td>
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<tr>
<td>Partially purified nuclear membranes</td>
<td>0.053 ± 0.018</td>
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<tr>
<td>Purified nuclear membranes</td>
<td>0.210 ± 0.039</td>
</tr>
<tr>
<td>Chromatin</td>
<td>0.0084 ± 0.002</td>
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</tbody>
</table>

![Fig. 2. Time course of the binding of insulin to nuclear membranes](http://www.jbc.org/)
maximal inhibition occurred at 40 to 60 ng/ml, and maximal inhibition occurred at 10,000 ng/ml (Fig. 3). On a molar basis, proinsulin had about 6 to 7% the activity of intact insulin, whereas desoctapeptide insulin had about 1 to 2% the activity of the intact hormone, potency values resembling those seen in both intact fat cell assays and liver plasma membrane binding assays (11, 12). Glucagon as well as growth hormone, prolactin, and thyrotropin did not influence the binding of insulin to nuclear membranes, indicating the hormonal specificity of the reaction (Fig. 3).

**Comparative Studies with Plasma Membranes**—Studies next were undertaken to compare the binding of insulin to both nuclear membranes and plasma membranes. On the basis of protein content, labeled insulin specifically bound to nuclear membranes about 20 to 30% as well as it bound to purified plasma membranes (Table II). A 5'-Nucleotidase activity, a marker for the plasma membrane, in liver and other tissues (13), was abundant in plasma membranes but was undetectable in purified nuclear membranes. This finding suggested a very low degree of contamination, if any, by plasma membranes of the nuclear membrane preparation used in this study.

Scatchard plots (11, 14, 15) of binding data for nuclear membranes indicated that the ratio of bound to free hormone was not a linear function of the hormone bound (Fig. 4). Data of this type also are seen in studies of insulin binding to plasma membranes, and may be interpreted as indicating the presence of two separate orders of binding sites (11, 14, 15). When a multiple site analysis is used in studies of nuclear membranes, two major orders of binding sites are also discerned: a high affinity site having a $K_d$ of 0.04 to 0.08 nM and a binding capacity of 1.0 to 2.0 pmol/mg of protein; and a lower affinity site having a $K_d$ of 65.6 ± 11.8 nM and a binding capacity of 2.7 ± 0.6 pmol/mg of protein. When plasma membranes are incubated under the same conditions (Fig. 5), two different orders of binding sites are seen: a high affinity site having a $K_d$ of 0.41 ± 0.04 nM and a binding capacity of 0.28 ± 0.02 pmol/mg of protein, and a lower affinity site

### Table II

<table>
<thead>
<tr>
<th>Membrane</th>
<th>5'-Nucleotidase activity</th>
<th>Insulin</th>
<th>Glucagon</th>
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</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>1.0 ± 0.02</td>
<td>0.8 ± 0.02</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Plasma</td>
<td>3.8 ± 0.04</td>
<td>12.3 ± 0.18</td>
<td>1300</td>
</tr>
</tbody>
</table>

*5'-Nucleotidase was also undetectable in purified nuclei.*
The pH optimum of insulin binding to nuclear membranes is found to be a plateau between pH 6.5 and 7.25 (Fig. 6). In contrast, under the same conditions, the pH optimum of insulin binding to plasma membranes is at pH 7.8 to 8.0 (11, 14, 15); and an enhanced dissociation, by unlabeled insulin, of previously bound labeled insulin (11, 14, 16, 17). The latter finding indicates the presence of negative cooperativity (site-site interactions) (16, 17). Accordingly, studies were undertaken to determine whether these characteristics were present in nuclear membranes.

The pH optimum of insulin binding to nuclear membranes was found to be a plateau between pH 6.5 and 7.25 (Fig. 6). This observation was made with two buffer systems, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and Tris. In contrast, under the same conditions, the pH optimum of insulin binding to plasma membranes was a sharp peak at pH 8.0 (Fig. 6).

The binding of insulin to plasma membranes was enhanced nearly 3-fold by increasing the NaCl concentration from 0.12 to 2 M (Fig. 7). In contrast, increasing the concentration of NaCl did not increase the binding of insulin to nuclear membranes (Fig. 7).

After incubating 125I-insulin with either nuclear membranes or plasma membranes, followed by a 100-fold dilution in insulin-free buffer, dissociation of the labeled insulin could be demonstrated (Fig. 8). The addition of unlabeled insulin enhanced the rate of dissociation of labeled insulin from plasma membranes, but, in contrast, unlabeled insulin had no effect on the rate of dissociation of labeled insulin from nuclear membranes (Fig. 8).

The present studies suggest that nuclear membranes are the major site of insulin binding to the cell nucleus. They further demonstrate that specific, high affinity binding sites for insulin are present on highly purified nuclear membranes. Several independent lines of evidence indicate that the binding of insulin to the nuclear membrane is not due to contamination of these preparations with plasma membranes.

First, both morphologic and enzymatic studies fail to reveal contamination. We can find no electron microscopic evidence for contamination of either the intact nuclei or the nuclear membranes employed herein by other cellular organelles, including plasma membranes. Also, purified nuclear membranes prepared by this method are reported to be devoid of NaKATPase (7), an enzyme located only on the plasma membrane. In addition, 5-nucleotidase, another marker enzyme for the plasma membrane (3), was undetectable in both nuclei and nuclear membranes.

Second, antisera from patients with extreme insulin resistance and acanthosis nigricans react with the insulin binding sites on plasma membranes to inhibit the binding of insulin.
Insulin Binding to Nuclear Envelope

(18). Under conditions where 90% of the binding of insulin to plasma membranes is inhibited, only 10 to 20% of the binding of insulin to nuclear membranes is inhibited (19). These studies strongly suggest that the binding sites for insulin on nuclear membranes are immunologically distinct from those on the plasma membranes.

Third, nuclear membranes bind glucagon but to a lesser extent than insulin. In contrast, plasma membranes bind glucagon 3 to 6 times more strongly than insulin. On one hand, based on this comparative binding data, it is possible that there is a 10 to 20% contamination of the nuclear membranes with plasma membranes. On the other hand, since highly purified rat liver nuclei contain glucagon-sensitive adenylate cyclase (20), we believe that it is more likely that the glucagon binding observed herein is an intrinsic property of nuclei and nuclear membranes.

Finally, direct comparative data indicate that the insulin binding sites on nuclear membranes have properties that are different from those on plasma membranes. Scatchard analysis indicates that, expressed per mg of protein, nuclear and plasma membranes have similar total binding capacities but different binding affinities. If the binding of insulin to nuclear membranes was due to contamination of this preparation with plasma membranes, one would expect to see similar binding affinities and different binding capacities. Even more important, the insulin binding sites on nuclear membranes show neither negative cooperativity nor enhancement of binding with 2 M NaCl, two major characteristics of the insulin binding sites on plasma membranes. Finally, the optimum pH for insulin binding is different in nuclear and plasma membranes.

The aforementioned studies suggest that the insulin binding sites on nuclear and plasma membranes are two different proteins. It is possible, however, that they are the same protein but that they behave differently because of the different environments of the nuclear and plasma membranes.

To what portion of the nuclear envelope insulin binds is unknown. In contrast to the plasma membrane, the nuclear envelope is a double-layered structure composed of an outer and an inner nuclear membrane (8, 21–23). Unfortunately, at present, it is not possible to separate and isolate these two nuclear membranes from each other (8, 21–23). It has been reported, however, that treatment of intact nuclei with detergents, such as Triton X-100, will remove much of the outer nuclear membrane but will not remove the inner nuclear membrane (23–25). Previously, we have reported that nuclei prepared by a modification of the method of Hymer and Huff (which employs Triton X-100) retain their ability to bind insulin (2). This finding suggests that the inner nuclear membrane may contain most of the insulin binding sites. In addition, we also reported that these detergent-washed nuclei do not bind glucagon (2). This finding also would suggest that the glucagon binding sites are on the outer nuclear membrane. More studies, however, will be necessary to clarify these points.

Insulin has multiple effects on target cells (26–29). These range from very rapid effects on plasma membrane transport to delayed effects on the synthesis of macromolecules (26–29). The mechanism whereby insulin exerts its long term effects, such as on the synthesis of DNA, RNA, and protein (26–29), is unknown. Studies from both this laboratory (2, 3) and others (9, 31) indicate that nuclei contain specific binding sites for insulin. In addition, we find that, after incubating intact human cultured lymphocytes with 125-I-insulin followed by either subcellular fractionation or autoradiography, it can be demonstrated that 10 to 30% of the total cellular insulin content enters the intact cell and then binds to its nucleus (1, 3). Others also have either observed or deduced that insulin can enter the intact cell (31, 32). Although the exact biological importance of these intracellular binding sites is unknown, on the basis of these data, we have suggested the possibility that insulin may exert certain of its intracellular effects by entering target cells and then binding to intracellular structures, such as the nuclear envelope (14, 29, 31).

This membrane structure is unique to eukaryotic organisms and undoubtedly plays an important role in regulating nuclear function. To date, however, no specific role for the nuclear envelope has been clearly demonstrated. Several investigators have suggested that one function of the nuclear envelope is to regulate the transport of materials into and out of the cell nucleus (21–23, 25). Since insulin regulates transport at the cell surface, it is speculated that one role of insulin in the cell interior may be to regulate transport at the nuclear surface.

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