Terbium, a Fluorescent Probe for Insulin Receptor Binding

Evidence for a Conformational Change in the Receptor Protein Due to Insulin Binding

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SUMMARY

Terbium (Tb^{3+}), an ion of the lanthanide series that has been used as a fluorescent probe for calcium (Ca^{2+}) binding sites in proteins, binds to the proteins in both solubilized and purified human placental insulin receptor preparations. Tb^{3+} fluorescence was determined directly and the effect of insulin binding on Tb^{3+}-enhanced fluorescence was studied without the need to separate bound and free ligands. Tb^{3+} behaved similarly to, but was more potent (50-fold) than, Ca^{2+} in increasing the insulin bound to its receptor.

When insulin bound to its receptor, the Tb^{3+} fluorescence of the receptor preparation decreased. When various insulin analogues were tested, the decrease in Tb^{3+} fluorescence was proportional to the biologic activity of the insulin analogues. In addition, Tb^{3+} could be displaced from insulin-sensitive sites by Ca^{2+}, indicating that there were Ca^{2+} (and Tb^{3+}) binding sites on or near the insulin receptor. These sites, when filled, were responsible for the increased insulin receptor affinity. The decrease in Tb^{3+} fluorescence after insulin binding may be indicative of a conformational change in the insulin receptor precipitated by the binding of insulin. This conformational change may be related to the release of Ca^{2+} by insulin binding, is associated with a decrease in insulin receptor affinity, and suggests that an allosteric mechanism involving both Ca^{2+} and insulin binding sites may be responsible for the observed changes in insulin receptor affinity. DIABETES 1984; 33:1106–11.

The activation of glucose transport in vitro by insulin in adipocytes and muscle,[1–4] the stimulation of protein synthesis by insulin,[3] and the phosphorylation of the insulin receptor in adipocytes[5] all require the presence of Ca^{2+} in the external medium for maximal activity. In fact, many cellular processes require Ca^{2+} or are modulated by the presence of Ca^{2+}. Therefore, it has been proposed that Ca^{2+} plays an essential role in the control of cellular function.[4,6,7]

The binding of insulin to its cell membrane receptor was increased by Ca^{2+} and, conversely, when insulin was bound to its receptor, Ca^{2+} was released from the membrane (in placenta,[8] rat liver,[9] and rat adipocytes[10]). This decrease in bound Ca^{2+} subsequent to insulin binding was proportional to the biologic activity of the insulin analogue tested.[8] Furthermore, the decrease in bound Ca^{2+} was also correlated with the decreased insulin receptor affinity, which suggested that these phenomena were biologically interdependent.[8] Ca^{2+} decreased insulin dissociation from the membrane and solubilized receptor,[9] a result that is consistent with an affinity change rather than unmasking of receptor sites and substances capable of altering bound Ca^{2+} reduced insulin receptor affinity in both membrane and solubilized receptor preparations.[11]

Ions of the lanthanide series have been used as probes for Ca^{2+} binding sites and one of these, Tb^{3+} (Terbium), when bound near certain aromatic amino acids, has the special property of luminescence when bound to certain proteins and biologic membranes.[12–19]

In the present study, we have examined the effect of insulin binding on the enhanced Tb^{3+} fluorescence of the Triton X-100 (TX-100) solubilized and purified human placental insulin receptor preparation. Using this fluorescent property, we were able to investigate the similarities of the Ca^{2+} and Tb^{3+} binding sites that were affected by insulin binding.

MATERIALS AND METHODS

Membrane preparation and incubation conditions for insulin binding. The microsomal membrane preparation used for insulin binding, $^{45}Ca^{2+}$ binding, and Tb^{3+}-enhanced fluorescence studies was prepared from human placenta as described previously.[8,20] Membrane preparations were solubilized with 0.01 ml TX-100/20 mg membrane protein, cen-

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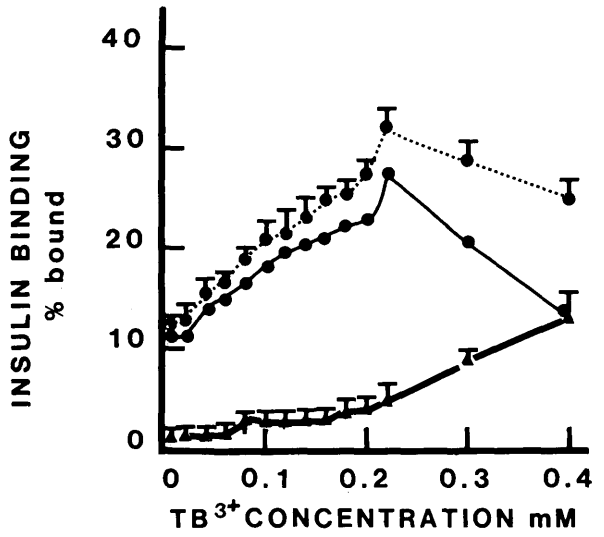
trifuged at 10,000 × *g*, and the supernatant diluted to a final protein concentration of 1–2 mg/ml. Incubation of membrane and solubilized receptor preparations for ^{125}I-insulin binding was performed in 0.3 ml of buffer A (50 mM Hepes-NaOH [Calbiochem, La Jolla, California], pH 7.0, plus 0.25% bovine serum albumin [BSA, fraction V RIA grade, Sigma, St. Louis, Missouri]) to which Ca^{2+} (0–6 mM) or Tb^{3+} (0.01–0.4 mM) and insulin (1×10^{-11}, -6×10^{-6} M) had been added. Incubation was for 1 h at 22°C and the bound and free hormone were separated by centrifugation or charcoal adsorption.[20] Membrane receptor (13.5 mg/ml) was also incubated for 1 h at 37°C with 1 mg/ml trypsin (192 U/mg, Worthington Biochemical Corporation, Freehold, New Jersey). It was then centrifuged at 13,000 × *g*, washed 3 times in buffer A, then solubilized in 0.5% TX-100 and diluted for



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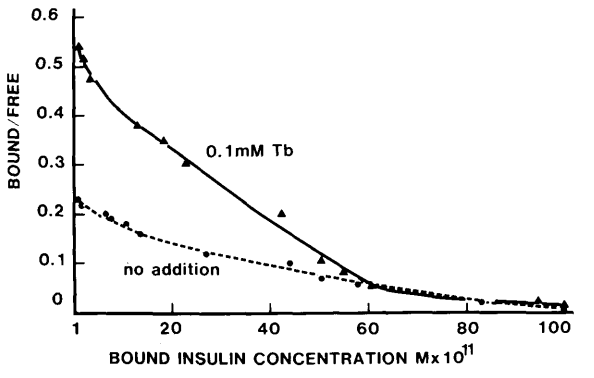

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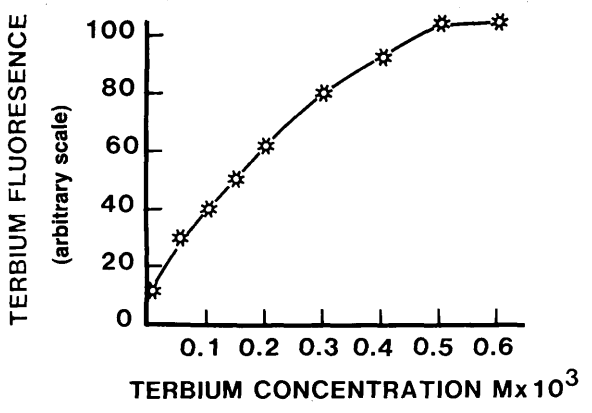
FIGURE 1. (A) Membrane protein (136.5 μg/tube) was incubated in buffer A containing tracer ^{125}I-insulin (2×10^{-11} M) and increasing concentrations of Tb^{3+}. Incubation was for 1 h at 22°C. Total (●----●), specific (●——●), and nonspecific binding (▲) were determined in the presence and absence of 2.5×10^{-6} M insulin, and bound and free hormone were separated by centrifugation.[22] The results are the means of two experiments ± 1 SD (N = 8 for each point). (B) Insulin displacement curves were carried out in the presence and absence of 0.12 mM Tb^{3+}. Incubation (150 μg membrane protein/tube) was for 1 h at 22°C in a total volume of 0.3 ml (N = 6). Bound and free hormone was separated by centrifuging the membrane pellet at 10,000 × *g* in a Sorvall RC2-B high-speed centrifuge. Scatchard plots were analyzed according to DeMeyts.[23]



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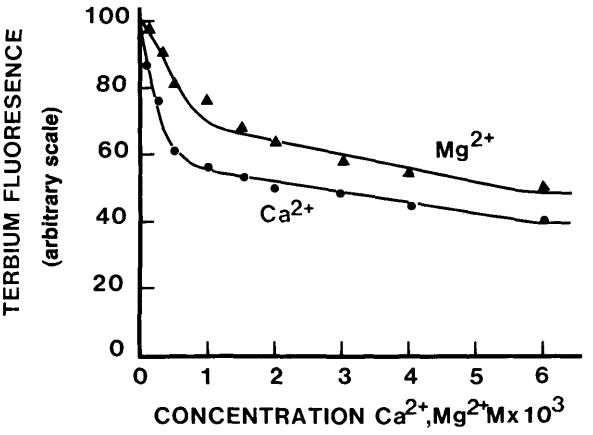

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FIGURE 2. (A) One milliliter of the membrane preparation (33 mg/ml) was solubilized with 16.5 μl of TX-100 for 1 h at 4°C, then centrifuged (10,000 × *g*) and the supernatant diluted with 19 ml of Hepes-NaOH buffer, pH 7.4. Increasing concentrations of Tb^{3+} were added to 0.1 ml of the solubilized receptor preparation and the final volume was made up to 0.3 ml with buffer A. Fluorescence was set at 100% for 0.6 mM Tb^{3+} and the fluorescence at lower concentrations was determined as a percentage of that value. Because of the arbitrary scale, results shown are for a single experiment, but superimposable curves were obtained in repeated experiments (N = 12). The coefficient of variation between experimental points was <2%. (B) The receptor preparation (13.5 mg/ml protein) was solubilized with TX-100, centrifuged, and the supernatant diluted to 9 ml with buffer A. Each incubation had a total volume of 0.4 ml with 220 μg of protein/tube. The final Tb^{3+} concentration was 0.1 mM. Fluorescence was measured after incubation at 22°C for 5 min in the presence of Tb^{3+} and Ca^{2+} or Mg^{2+}. Results are the means of a single experiment, and each point represents the mean of 6 determinations with a coefficient variation of 2%.

use in the insulin binding assay and the displacement of Tb^{3+} by insulin.

A purified insulin receptor preparation was prepared according to our previously described method[20] and the effect of Ca^{2+} and Tb^{2+} on insulin binding to this preparation was examined.

Insulin standards of porcine insulin (single peak, Eli Lilly and Company, Indianapolis, Indiana), triacetyl insulin (Hoechst AG, Frankfurt/Main, FRG), bonito insulin (Novo Industries A/S, Copenhagen, Denmark), porcine proinsulin (Novo), and diamino-suberoyl insulin (Hoechst AG) were prepared in buffer A. These standards were added to the ^{125}I-insulin binding assays in increasing concentration and the amount of ^{125}I-insulin bound by the membrane was de-

termined. Scatchard plots of insulin displacement curves were analyzed according to DeMeyts[21] because of the previous demonstration of negative cooperativity in this tissue.[8,11]

Tb^{3+}-enhanced fluorescence. Polyethylene plastic tubes (3 ml, NUNC minisorp, Kamstrup, Denmark) were used for all incubations and contact with glass containers or vessels was avoided.[16] The Tb^{3+} solutions were prepared by dissolving sealed vials of anhydrous $TbCl_3$ (Koch-Light, Colnbrook, United Kingdom) in plastic volumetric flasks and were stored at a concentration of 50 mM in sealed plastic bottles. Tb^{3+} standards, in which the absolute concentration had been determined by ion exchange chromatography and back-titration,[16] were compared in the absence of protein to the above solutions by proportional fluorescence of directly activated Tb^{3+} (excited at 353 or 368 nm).

Fluorescence measurements were made at 22°C in a Perkin-Elmer MPF-2A fluorescence spectrophotometer using Raman cylindrical cuvettes with a total volume of 0.5 ml. A spectral band width of 10 nm was used for excitation and emission. Titration of solubilized, membrane, or purified insulin receptor preparation with $TbCl_3$ was made by sequential addition of 2–10 μl of stock solutions of either 1 or 10 mM of Tb^{3+}.

Fluorescence emission in the presence of protein was monitored at 545 nm using a 430-nm filter to screen incident excitation light. Excitation of the Tb^{3+}-enhanced fluorescence was maximal at 291 nm for both the solubilized and membrane receptor preparation. Competitive binding between Ca^{2+}, Mg^{2+}, and Tb^{3+} was determined by the fluorescence changes after incubation for 5 min at 22°C in 0.3 ml buffer A. However, direct titration by the sequential addition of small volumes of Ca^{2+} or Mg^{2+} to cuvettes containing the receptor preparation and Tb^{3+} was also carried out, without preincubation. Insulin standards for the displacement of Tb^{3+} were the same as those described above. The time course of the decrease in Tb^{3+} fluorescence in response to insulin binding was monitored in solubilized and purified receptor preparations. Excitation at 280 nm was also carried out and the ratio of peak heights at 545 nm for several preparations was determined. This allowed identification of the amino acid responsible for the Tb^{3+}-enhanced fluorescence.

Human growth hormone (hGH, 0.1 IU/mg, Commonwealth Serum Laboratories, Melbourne, Victoria, Australia), prolactin (ovine, NIADDK-NIH, Bethesda, Maryland), glucagon (crystalline porcine, Eli Lilly and Company), and ACTH (105 U/mg, Sigma) standards were prepared in buffer A. They were added to the solubilized receptor preparation at final concentrations of 2.4×10^{-8} M hGH, 1.0×10^{-8} M prolactin, 2.1×10^{-11} M glucagon, and 1.0×10^{-7} M ACTH.

Gel chromatography of the insulin receptor. Gel chromatography of the solubilized receptor preparation was carried out on a column of Sepharose 6B (Pharmacia, Piscataway, New Jersey, 29 × 1.5 cm) in 50 mM Hepes, pH 7.5, 0.1% TX-100, and 0.05% sodium azide at 22°C. The total column volume was 205 ml and 2.5-ml fractions were collected. Concentrated solutions of solubilized receptor preparations were loaded onto the column[24] and the optical density of each fraction was determined at 254 nm. Aliquots of each fraction were removed for the determination of Tb^{3+} binding by fluorescence (0.5 ml) and specific insulin binding (0.1 ml).

RESULTS

^{125}I-insulin binding to the solubilized placental receptor preparation in the presence of Tb^{3+} was enhanced over the concentration range of 0.02–0.2 mM Tb^{3+}. At higher concentrations, nonspecific binding increased without a further increase in insulin binding (Figure 1A). This was presumably due to precipitation of the ^{125}I-insulin by Tb^{3+}. The increased specific insulin binding was due to an increase in receptor affinity and not to a change in total receptor number (Figure 1B). Analysis of the Scatchard plots of the insulin displacement curves gave: Ke 2.2×10^8 M^{-1}, Kf 1.3×10^8 M^{-1}, and Ro 98×10^{-11} M in the absence of Tb^{3+}, and Ke 5.4×10^8

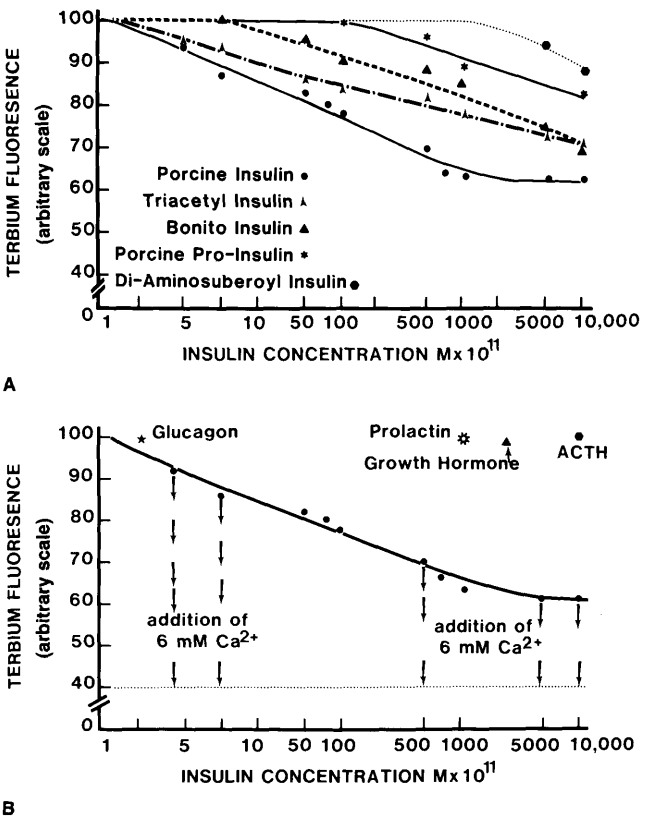

FIGURE 3. (A) Displacement of Tb^{3+} by insulin analogues. Solubilized membrane receptor preparation (150 μg protein/tube) was incubated in buffer A at pH 7.4 in a total volume of 0.4 ml. Insulin or its analogues were added in 0.05 ml of buffer A at the concentration indicated. The Tb^{3+} concentration was 0.038 mM. Fluorescence was determined after incubation for 1 h at 22°C. Results are shown as the mean of 4 separate determinations with a maximum variation of 2% in fluorescence measurements. (B) Displacement of Tb^{3+} by Ca^{2+} and insulin. Porcine insulin standard was used to decrease the Tb^{3+} (0.03 mM) fluorescence of the solubilized receptor preparation (132 μg protein/tube). This was then incubated for 1 h at 22°C and the fluorescence determined. Ca^{2+} (6 mM final) was added to the cuvette in a volume of 0.01 ml, the solution was mixed, and fluorescence redetermined. The decrease in fluorescence after the addition of Ca^{2+} did not exceed that seen with 6 mM Ca^{2+} alone (represented by the dotted line) at all insulin concentrations tested. No decrease in fluorescence was seen in samples incubated with glucagon, prolactin, hGH, or ACTH. Each point represents the mean of 2 experiments carried out in triplicate, with variation between replicates of <2%.

M^{-1}, Kf 1.3 × 10^8 M^{-1}, and Ro 98 × 10^{-11} M in the presence of Tb^{3+} (Figure 1B), where Ke = affinity of the insulin receptor when all sites are empty, Kf = affinity of the insulin receptor when all sites are full, and Ro = total receptor concentration. The purified insulin receptor also demonstrated increased specific insulin binding in the presence of 5 mM Ca^{2+} from 5.7 ± 0.7 to 21.84 ± 0.64%. Tb^{3+} at 0.2 mM increased specific insulin binding from 6.83 ± 0.34 to 15.5 ± 1.06%. Saturation of the Tb^{3+} binding sites by fluorescence measurements occurred at 0.5 mM and was half-maximal at 0.15 mM (Figure 2A). Ca^{2+} and Mg^{2+} reduced fluorescence by 60% and 52%, respectively (Figure 2B). The decrease in Tb^{3+} fluorescence (and, therefore, binding) was half-maximal at 0.4 mM for Ca^{2+} and 0.8 mM for Mg^{2+} (Figure 2B). For both ions, a concentration of 6 mM was required to achieve maximal Tb^{3+} displacement (Figure 2B).

The decrease in Tb^{3+} fluorescence of the solubilized receptor preparation produced by insulin and its analogues was directly proportional to both concentration and biologic activity (Figure 3A), but represented only 60% of the Ca^{2+}-displaceable and 40% of the total Tb^{3+} binding sites. Half-maximal displacement of Tb^{3+} from the solubilized receptor preparation occurred with 3.5 × 10^{-10} M porcine insulin, 2.5 × 10^{-9} M triacetyl insulin, 2.5 × 10^{-8} M bonito insulin, 2.5 × 10^{-7} M porcine proinsulin, and 4 × 10^{-6} M diaminosuberoyl insulin. Maximal displacement with porcine insulin was only reached at 7.5 × 10^{-9} M. When insulin and Tb^{3+} were added together and the fluorescence monitored without preincubation, the time course of the decrease in fluorescence was consistent with the association rate of insulin with the solubilized receptor reported previously,[11] being half-maximal at 20 min and complete in 1.5 h at 22°C. Tryptic digestion of the membrane receptor preparation, followed by solubilization, reduced Tb^{3+} fluorescence by 50%, and abolished insulin binding and the ability of insulin to decrease the Tb^{3+} fluorescence (Table 1). No decrease by insulin of the Tb^{3+} fluorescence was seen with phospholipase-A[14] or in tryptic digests of membrane preparations (Table 1). The addition of insulin to the purified receptor preparation containing 0.2 mM Tb^{3+} resulted in a concentration and time-dependent decrease in fluorescence (Table 2).

The decrease in Tb^{3+}-enhanced fluorescence caused by insulin to the solubilized receptor was not additive to the displacement seen with 6 mM Ca^{2+} alone at any concentration of insulin tested. Other peptide hormones (ACTH, glucagon, growth hormone, and prolactin) at concentrations that caused maximal displacement of labeled hormone from their own receptors had no effect on the Tb^{3+}-enhanced fluorescence (Figure 3B).

The ratio of the peak heights for the Tb^{3+}-enhanced fluorescence of the receptor preparation when amino acids were excited at 291 or 280 nm was 1.38 ± 0.12 for four different preparations, indicating that tryptophan or ionized phenolate groups were responsible for the resonance energy transfer to Tb^{3+}.[17]

Gel chromatography of the solubilized insulin receptor on Sepharose 6B revealed that Tb^{3+} binding and insulin binding occurred in the same fractions (Figure 4), but other proteins that bound Tb^{3+} were also present in this preparation.

DISCUSSION

Bound Tb^{3+} has fluorescent properties at micromolar concentrations and can accept energy from suitably excited amino acids in proteins (indirect excitation). This energy is transmitted via radiationless energy transfer and Tb^{3+} emits light in the green 535–555 nm spectral region.[14] In this region, only the tail of the emission peaks of amino acids are found.[14] The ratio of peak heights for Tb^{3+}-enhanced fluorescence when the receptor preparation was excited at 291 and 280 nm indicates that either tryptophan or ionized phenolate groups may be involved in the resonance energy transfer.[14] This does not mean that these groups were responsible for Tb^{3+} binding, but, rather, that they were adjacent to a bound Tb^{3+} ion.

Tb^{3+} binding to solubilized human placental insulin receptor preparations was saturable (Figure 2A), and shared some common sites with Ca^{2+} and Mg^{2+} (Figure 2B). Like Ca^{2+}, Tb^{3+} increased insulin receptor binding by increasing receptor affinity for insulin (Figure 1A and B). Typical of the lanthanide ion series, Tb^{3+} was 50–100 times more potent than Ca^{2+} or Mg^{2+}, but it also interacted at sites that were unaffected by Ca^{2+} or Mg^{2+}.[18,19] These sites could be Na^+

TABLE 1
Tb^{3+}-enhanced fluorescence of the soluble receptor preparation derived from control and tryptic-digested membranes, demonstrating the loss of sensitivity to insulin*

Insulin concentration (× 10^{-11} M)	Fluorescence (arbitrary scale [0–100])		
	Control	Tryptic-digested	Phospholipase-A
0	100	50	100
5	90	48	100
10	80	47	100
50	77	46	100
75	77	50	100
100	72	48	100
250	66	50	100
500	60	48	100
Receptor (no Tb^{3+})	10	15	—
Tb^{3+}, buffer A	5	5	—

Values represent the mean of eight observations obtained in two separate experiments.
*The inability of insulin to affect the Tb^{3+} fluorescence of 50 mg of phospholipase is also shown as a control.

TABLE 2
Tb^{3+}-enhanced fluorescence of the purified receptor preparation, demonstrating the effect of time and insulin concentration

	Fluorescence (arbitrary scale [0–100])		
	Albumin	Receptor	
Time (min)	Insulin (1×10^{-6} M)	Insulin (2×10^{-9} M)	Insulin (1×10^{-6} M)
0	44	40	40
10	42	40	35
15	40	35	33
20	40	32	29
30	40	30	25
60	40	28	23
90	40	22	17

Values represent the mean of three observations in two separate experiments.

sites that are capable of binding Ca^{2+} loosely, and to which the lanthanide ions (with their increased affinity) appear to bind more avidly than Ca^{2+}.[19] Nevertheless, Ca^{2+} was more potent than Mg^{2+} (Figure 2B) in displacing Tb^{3+} from the majority of sites that were sensitive to divalent cations, especially those affected by insulin (Figure 3). The fact that tryptic digestion of the receptor preparation before solubilization lowered Tb^{3+} binding by 50% (as well as insulin binding and insulin displacement of Tb^{3+} binding) suggested that the Tb^{3+} binding sites were on proteins rather than on carbohydrates or phospholipids in the membrane.

Porcine insulin reduced Ca^{2+} [8] and Tb^{3+} bound to the placental receptor binding sites over a physiologic concentration range (1–200 μU/ml, 1–1400 $\times 10^{-11}$ M). The correlation between in vivo biologic potency of various insulin analogues[22,23] and their ability to displace Tb^{3+} from the receptor, established that the displacement was specific, and a direct consequence of insulin binding to its receptor. Additional evidence to support this concept was provided by the displacement of Tb^{3+} from the purified receptor. The insulin molecule itself did not contain a site that bound Tb^{3+} without fluorescence (thereby reducing the Tb^{3+} available for fluorescence with the receptor preparation), because insulin did not change Tb^{3+} fluorescence when added to tryptic-digested membrane, nor did it affect the fluorescence of a known Ca^{2+}-binding protein, phospholipase-A,[14] or albumin. Other peptide hormones at saturating concentrations for their own receptors had no effect on Tb^{3+} fluorescence in these insulin receptor preparations. Ca^{2+} and insulin appeared to displace Tb^{3+} from identical sites, because the displacement of Tb^{3+} caused by insulin was not additive to the displacement seen with 6 mM Ca^{2+} alone. Ca^{2+} did displace Tb^{3+} from sites other than those affected by insulin (Figure 3B), indicating the presence of Ca^{2+} binding sites that were not affected by insulin binding. These may be responsible for the high-molecular-weight fluorescence peaks seen in Figure 4 that do not coincide with the peak of receptor binding activity.

The human placental insulin receptor demonstrates negative cooperativity both in the membrane and solubilized form,[11,24] indicating the presence of a single site with high affinity that decreases in affinity proportional to the number of receptor sites occupied. The increased insulin binding seen in the presence of Tb^{3+} was therefore interpreted as an increase in the affinity of the insulin receptor and not as a change in receptor numbers for the high-affinity component. There are two possible explanations for the increase in insulin receptor affinity due to Tb^{3+} binding and the decrease in the Tb^{3+}-enhanced fluorescence on insulin binding to the receptor preparation: (1) Tb^{3+} (or Ca^{2+}) bound to the insulin binding site of the receptor and thereby changed the affinity for insulin, or (2) Tb^{3+} (or Ca^{2+}) bound to a site on or near the receptor, but not the binding site, and increased insulin receptor affinity. Insulin binding then caused a conformational change in the insulin receptor that was reflected either by a movement of the Tb^{3+} (and Ca^{2+}) binding site away from the aromatic amino acid residue responsible for the resonance energy transfer, or it released these ions from the site. A movement of greater than 10 Å is required for this decrease in fluorescence.[19] A conformational change in the insulin receptor itself[8] or a generalized conformational change of the membrane structure[9,25,26] in response to insulin binding has been proposed as the mechanism for the decrease in divalent cation binding. Such a conformational change in the receptor has been demonstrated by the increased sensitivity to tryptic digestion of the insulin receptor after insulin binding[27] and by radiation inactivation analysis.[28] Taken together, these observations suggest that an allosteric interaction between Ca^{2+} and insulin may regulate insulin receptor affinity. The possibility that Tb^{3+} bound to the insulin binding site of the receptor is unlikely because lanthanide ions, when bound at the active site of Ca^{2+}-binding proteins, have usually inactivated them due to the increased affinity of these ions for the Ca^{2+} sites.[19] Such proteins are grouped into "class 1," Ca^{2+}-binding proteins because of the inhibitory action of lanthanides. Therefore, insulin receptor appears to fall into the "class 2" of the Ca^{2+}-binding proteins in which lanthanide ions "yield proteins that function similarly and sometimes more effectively than when Ca^{2+} was bound to these proteins."[19] This distinction provided by lanthanide ion substitution between "class 1" and "class 2" Ca^{2+}-binding proteins is important in that it indicates whether Ca^{2+} is

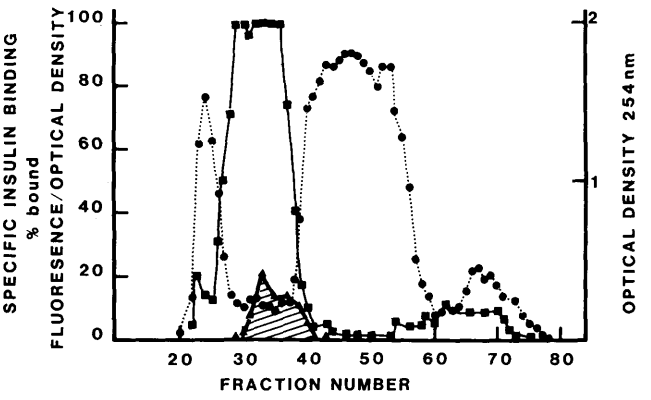

FIGURE 4. Chromatography of the solubilized insulin receptor preparation (240 mg protein) on Sepharose 6B. Fraction volume was 2.5 ml, fluorescence was determined in 0.1-ml aliquots, and results are expressed as an integer of the optical density of the sample (■). The optical density (●) was measured at 254 nm and specific insulin binding (▲, shaded area) was determined in 0.05-ml aliquots in each fraction in triplicate.

involved at the active site or at a site that is more remote and, therefore, more important structurally to the protein.[19] "Class 2" Ca^{2+}-binding proteins are more likely to demonstrate a conformational change upon ligand binding, and have a Ca^{2+} binding site remote from the active region.

Tb^{3+}-enhanced fluorescence occurs when energy transfer from the ligand to the ion can occur with greatest efficiency, and this is in the relative absence of water.[18] This would indicate that Tb^{3+} (or Ca^{2+}) was bound to the hydrophobic areas of the receptor, most likely those near the lipid bilayer of the membrane. When insulin is bound, these hydrophobic areas may become more exposed to water as a result of conformational change, and, hence, Tb^{3+} fluorescence is decreased.

The peak of insulin receptor binding after gel chromatography of the insulin receptor preparation and a peak of Tb^{3+} fluorescence are coincident (when corrected for protein concentration, Figure 4). This indicates that a receptor-associated protein does contain Tb^{3+} binding sites and presumably Ca^{2+} binding sites because of the similar effects of these ions on the receptor. The optical density of the fraction was determined at 254 nm because of the absorbance peak for TX-100 at 272 nm.[19] The high-molecular-weight peak preceding the receptor may be responsible for the Ca^{2+} and Tb^{3+} binding sites that were unaffected by insulin (insulin displaced only 60% of the Ca^{2+}-displaceable sites and Ca^{2+} decreased 60% of the Tb^{3+} fluorescence [Figure 3B]). Furthermore, the purified receptor preparation fluoresced in the presence of Tb^{3+}, and insulin binding decreased its fluorescence in a time- and concentration-dependent manner. Tb^{3+} appears to be capable of fulfilling the role of Ca^{2+} in most of its actions on the insulin receptor, i.e., enhancement of receptor affinity and displacement by hormone binding.

These studies suggest that the conformational state of the insulin receptor is both hormone- and Ca^{2+}-dependent. Changes in the conformational state can be monitored by changes in Tb^{3+} fluorescence, as Tb^{3+} appears to substitute for Ca^{2+} in its effects on insulin receptor affinity, insulin binding, and the displacement from the receptor consequent to insulin binding. The Tb^{3+}-enhanced fluorescence can be monitored without complicated separation techniques and provides a means whereby the allosteric regulation of insulin receptor affinity by Ca^{2+} or Tb^{3+} may be examined.

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