PURIFICATION OF PLACENTAL INSULIN RECEPTORS BY THE USE OF INSULIN-DEXTRAN, A SOLUBLE BIOSPECIFIC MACROMOLECULE

SUSUMU SUZUKI*, YOSHIKAZU SAKAMOTO, TAKESHI KUZUYA and YOKO
FUJITA-YAMAGUCHI**

Division of Endocrinology and Metabolism, Department of Medicine, Jichi Medical School, Minami-kawachi machi, Tochigi 329-04, and **Division of Biology, Molecular Genetics Section, City of Hope Medical Research Institute, Duarte, Calif., U.S.A.

ABSTRACT

A new method for purifying insulin receptors by the use of a soluble biospecific macromolecule, insulin-dextran, is described as an alternative means to conventional solid-phase affinity chromatography. Crude insulin receptors were prepared from plasma membrane fraction of human placenta, solubilized with Triton X-100, followed by ultra-centrifugation, affinity chromatography with concanavalin A Sepharose, and gel filtration with Sepharose 6B. The resulting preparation was reacted with insulin-dextran and then chromatographed on a Sepharose 6B column at pH 7.4 in the presence of Triton X-100 (0.1%). Insulin receptors were dissociated from insulin-dextran by acidification to pH 6.0 and purified by rechromatography on the same column at pH 6.0. By these procedures, 8,700-fold purification of insulin receptors was achieved from solubilized membrane fraction without significant changes in its affinity constants. The molecular weight of the receptors was estimated as about 300,000.

KEY WORDS insulin receptors / purification / placenta / insulin-dextran / soluble biospecific ligand-gel filtration method

Purification of insulin receptors from detergent-solubilized plasma membrane of rat liver, fat cells and human placenta has been described (1, 2, 8, 12, 13, 20). Affinity chromatography using insoluble agarose coupled with insulin or concanavalin A has been used as a chief means to purify insulin receptors. There are some problems, however, in using this insulin-bound solid matrix. Agarose has a relatively high ion exchange property, which may cause a considerable retention of non-specific proteins along with insulin receptors, and may contaminate the receptor preparation after elution. Technically, it is not easy to prepare stable insulin-agarose.

Free insulin may be released from insulin-agarose during experiments (9).

In the present communication, we describe an alternative method to ordinary affinity chromatography for purifying insulin receptors. In this method, a water-soluble dextran was used instead of agarose as a biospecific ligand. Insulin-linked dextran molecules have insulin-like biological activity and do not liberate free insulin during incubation with adipose tissue (16, 17). Crude insulin receptor fraction was first incubated with this insulin-dextran, yielding a receptor-insulin-dextran complex which was easily separated by gel filtration from other proteins with molecular weight similar to insulin receptors. The same principle was successfully used by Hubert *et al.* (7) for the purification of

*Present address: The Third Department of Medicine, Tohoku University, Sendai 980, Japan

estrogen receptors from calf uterus. This report is the first application of a soluble bio-specific carrier to purification of the receptors of a peptide hormone.

MATERIALS AND METHODS

Monocomponent pork insulin was obtained from Novo Laboratory (Copenhagen). ^{125}I -insulin ($236\text{ }\mu\text{Ci}/\mu\text{g}$) was purchased from Dainabot Radioisotope Laboratory (Tokyo). Dextran T40 and Sepharose 4B and 6B were purchased from Pharmacia Fine Chemicals (Uppsala). Biobeads SM-2 were obtained from Bio-Rad Laboratories (Richmond, Calif.). Concanavalin A and α -methyl mannoside were obtained from Sigma (St. Louis, Missouri), and Triton X-100 and polyethylene glycol 6,000 and 20,000 from Wako Pure Chemicals (Tokyo).

Preparation of Insulin-Dextrans and Concanavalin A Sepharose

Dextran T40 was activated by cyanogen bromide and coupled to ^{125}I -insulin (insulin-dextran I), or pork monocomponent insulin plus ^{125}I -insulin (insulin-dextran II), as described elsewhere (16). Briefly, 400 mg cyanogen bromide in 10 ml was added to 500 mg Dextran T40 and pH was maintained at 8.5–10.5 for 6 min by the addition of 1 N NaOH, and then 500 ml acetone was added. The precipitate, the activated Dextran T40, was collected on a filter paper. The activated dextran was reacted with 3.6 mg ^{125}I -insulin or 2 mg pork monocomponent insulin plus 3.6 ng ^{125}I -insulin in 6 ml 0.1 M NaHCO_3 (pH 9.0) for 12 hr at 4°C with continuous stirring. The reaction product was lyophilized. Insulin-dextran I and II preparations had a mean molecular weight of about 150,000. They were purified by successive gel filtrations on a column of Sephadex G-75 ($2.5 \times 50\text{ cm}$) and a column of Sepharose 6B ($1.5 \times 90\text{ cm}$) with 3 M acetic acid as eluant. By this procedure, insulin-dextran preparations were freed of unbound ^{125}I -insulin. The amount of insulin bound to dextran was estimated to be 24 pg ^{125}I -insulin per g for insulin-dextran, and 12.3 μg insulin per g for insulin-dextran II. Specific activity of ^{125}I -insulin was 5.7 and 5.2 μCi per g for insulin-dextrans I and II, respectively. The same lots of insulin dextrans were used throughout this study.

Twenty milliliters of Sepharose 4B was activated by cyanogen bromide similarly and coupled to 100 mg concanavalin A in 40 ml 0.1 M NaHCO_3 (pH 9.0) for 12 hr with continuous

stirring according to method of Lloyd (10). The amount of concanavalin A bound to Sepharose 4B was 4.98 mg per ml. About 1 mg of soluble plasma membrane was adsorbed to 1 ml of concanavalin A-Sepharose (Con A Sepharose) by a batch method.

Preparation and Solubilization of Placental Plasma Membrane

Fresh normal human placentas were collected after full term vaginal delivery, and stored frozen at -20°C . Placental plasma membrane was prepared by differential centrifugation as described by Posner (15). Frozen tissue was cut into small pieces and homogenized in 0.3 M sucrose in a glass homogenizer with a Teflon pestle. The homogenate was filtered through a gauze and centrifuged. After centrifugation for 15 min at 600 *g*, the supernatant was centrifuged for 20 min at 15,000 *g*. The supernatant after this centrifugation was again centrifuged at 100,000 *g* for 60 min. The pellet (crude plasma membrane fraction) was solubilized in 1% (v/v) Triton X-100 in 50 mM Tris-HCl buffer (pH 7.4), in a concentration of 5 mg protein per ml buffer. After gentle stirring for 30 min at room temperature, insoluble material was removed by centrifugation at 200,000 *g* for 60 min at 4°C . Excess Triton X-100 was removed from the sample by a gentle stirring with 0.3 g Biobeads SM-2 per ml for 30 min at 4°C according to the method of Holloway (6). It was centrifuged again at 200,000 *g* for 60 min and the resultant supernatant (soluble membrane fraction) was used as the source of soluble insulin receptors.

Binding of Insulin-Dextran I to Soluble Membrane Fraction

As a pilot experiment, soluble membrane fraction (200 μg of protein) was incubated with 1 mg insulin-dextran I in 2 ml 50 mM Tris-HCl buffer (pH 7.4), containing 0.1% Triton X-100, in the presence and absence of excess unlabelled pork insulin (10 $\mu\text{g}/\text{ml}$), ACTH (1 $\mu\text{g}/\text{ml}$), or glucagon (1 $\mu\text{g}/\text{ml}$). Incubation was carried out for 12 hr at 4°C . Receptor-bound insulin-dextran I was separated from free insulin-dextran I by gel filtration on a Sepharose 6B column. The incubation mixture was applied to a Sepharose 6B column ($1.5 \times 90\text{ cm}$), eluted with the same buffer at a flow rate of 8 ml/hr, and 2.3 ml fractions were collected. The radioactivity of each fraction was measured by an autogamma spectrometer. Fractions which contained re-

ceptor-insulin-dextran complex were collected and concentrated against polyethylene glycol 20,000. Then, dissociation of insulin-dextran from the receptors was tested by acidification of the sample to pH 6.0 or by the addition of excess unlabelled insulin (10 μ g/ml). For the acidification experiment, a Sepharose 6B column was equilibrated with 50 mM Tris-HCl buffer (pH 6.0) and 0.1% Triton X-100 at least for 12 hr before use. Tris-HCl solution has little buffering capacity at this pH, but it was used because it worked satisfactorily and the assay of insulin binding was carried out in Tris-HCl buffer (pH 7.4) throughout this study. The fraction containing receptor-insulin-dextran complex was adjusted to pH 6.0 by the addition of 1 N HCl, and kept at 4°C for 12 hr, and then chromatographed on this column and eluted with the same buffer in 2.3 ml fractions. Radioactivity of each fraction was counted.

Assay of Insulin Binding and Protein Concentration

Each sample was incubated with 182 pg of 125 I-insulin in the presence and absence of excess unlabelled insulin (10 μ g/ml) in a total volume of 0.4 ml for 12 hr at 4°C. The assay buffer was 50 mM Tris-HCl buffer (pH 7.4), 0.1% bovine serum albumin for particulate membrane, and 0.1% Triton X-100 was added to the same buffer for soluble receptors. 125 I-insulin bound to membranes was collected by centrifugation at 3,000 rpm for 30 min at 4°C. For soluble receptors, polyethylene glycol 6,000 (final concentration, 10%) and dog crude γ -globulin (final concentration, 0.04%) was added, and the mixture was centrifuged at 3,000 rpm for 30 min at 4°C (4). In some experiments, the bound/free ratio of 125 I-insulin was estimated by gel filtration in 0.1% Triton X-100, which gave essentially identical results with those obtained by polyethylene glycol precipitation. Protein concentration of samples was measured according to the method of Lowry *et al.* (11), using bovine serum albumin as standard.

Affinity Chromatography with Concanavalin A Sepharose

Con A Sepharose column of 20 ml volume was equilibrated with 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM CaCl_2 , 2 mM MgCl_2 , and 0.1% Triton X-100. Soluble membrane fraction (2.1 mg/ml, 230 ml) was applied to this column and washed with 130 ml of the buffer at

4°C, and then the bound materials were eluted in one step by 40 ml 0.3 M α -methyl-D-mannoside dissolved in the same buffer. Fractions, 4.6 ml each, were collected, and a 100 μ l aliquot of each fraction was taken and assayed for specific insulin binding activity and protein concentration. Fractions with high insulin-binding capacity (Con A fractions) were pooled and concentrated against polyethylene glycol 20,000.

Sepharose 6B Gel Filtration

A 1.5 $\times$ 90 cm column of Sepharose 6B was equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and 0.1% Triton X-100. The Con A fraction was applied to the Sepharose 6B column and eluted with the same buffer. Fractions, 2.3 ml each, were collected at a flow rate of 8 ml/hr. An aliquot of each fraction was assayed for specific insulin binding and protein concentration. Fractions with high insulin-binding capacity (Sepharose 6B fraction) were pooled and concentrated against polyethylene glycol 20,000.

Gel Filtration of Soluble Receptor-Insulin-Dextran Complex

Sepharose 6B fraction was gently mixed with Biobeads SM-2 at 4°C for 30 min, and then the Biobeads were removed by centrifugation. With this procedure, concentration of Triton X-100 was reduced below 0.1%. This step was necessary, because Triton X-100 interferes with binding of insulin to the receptors at concentrations higher than 0.2%. The biobeads-treated solution was then incubated with 2 mg of insulin-dextran II, which bound 44 pg of 125 I-insulin and 24.5 μ g of unlabeled insulin, in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 for 12 hr at 4°C. The incubation mixture was applied again to the Sepharose 6B column and eluted with the same buffer in 2.3 ml fractions. Radioactivity, specific insulin binding, and protein concentration were assayed for each fraction. Several fractions which contained receptor-insulin-dextran complex were pooled and concentrated against polyethylene glycol 20,000, and then acidified to pH 6.0 with 1 N HCl, and incubated for 12 hr at 4°C. The same Sepharose 6B column was equilibrated overnight with 50 mM Tris-HCl buffer (pH 6.0) containing 0.1% Triton X-100, and the acidified fraction was applied to this column and eluted with the same buffer (pH 6.0). Fractions, 2.3 ml each, were collected and neutralized to pH 7.4 by addition of 1 M Tris solution. Each

fraction was measured for radioactivity, insulin-binding capacity, and protein concentration. Fractions with high insulin-binding capacity were pooled and concentrated against polyethylene glycol 20,000 (purified insulin receptor fraction).

RESULTS

Binding of Insulin-Dextran I to Soluble Insulin Receptors

Fig. 1a shows the elution profile of the reaction mixture of insulin-dextran I and soluble recep-

Fig. 1 Gel filtration of solubilized plasma membrane fraction of human placenta after incubation with insulin-dextran I. a: Solubilized plasma membrane fraction and insulin-dextran I were incubated in the presence (—●—) and absence (—○—) of excess unlabeled pork insulin (10 µg/ml), chromatographed on a Sepharose 6B column, and eluted with 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100. Each fraction was counted for ^{125}I . Peaks A and B represent the receptor-insulin-dextran complexes and peak C represents free insulin-dextran I. b: The reaction mixture of solubilized plasma membrane fraction and insulin-dextran I was adjusted to pH 6.0 with 1 N HCl, and chromatographed on a Sepharose 6B column equilibrated with 50 mM Tris-HCl buffer (pH 6.0) containing 0.1% Triton X-100. Only one peak (peak C) of radioactivity of free insulin-dextran I was observed.

tors on a Sepharose 6B column. It provides a basis for the purification of insulin receptors by means of soluble biospecific ligand-gel filtration. There were two peaks (A and B) of radioactivity, one of which (peak A) corresponded to the void volume. Addition of excess unlabeled insulin to the reaction mixture caused the disappearance of peak A and peak B, and the appearance of a new peak of radioactivity (peak C). Therefore, peak A and peak B seem to represent the receptor-insulin-dextran I complex, and peak C corresponds to free insulin-dextran I. ACTH and glucagon at a concentration of 1 $\mu\text{g}/\text{ml}$ did not displace peaks A and B (data not shown). The binding of insulin-dextran I to soluble receptors at pH 6.0 as examined by a similar method was less than 5% of that at pH 7.4. Acidification of the incubation mixture to pH 6.0 almost completely dissociated the receptor-insulin-dextran complex as shown in Fig. 1b.

Preparation and Purification of Insulin Receptor

From five human placentas (2,160 g), 1.28 g of crude plasma membrane was obtained. Treatment with 1% Triton X-100 for 30 min solubilized 41% of the protein and 45% of the insulin-binding activity of this membrane fraction. This solubilized material was used for the subsequent purification of insulin receptors, and was first subjected to affinity chromatography using a Con A Sepharose column.

Affinity Chromatography with Concanavalin A Sepharose

Fig. 2 shows the elution pattern of insulin receptors and protein. Since Triton X shows absorbance at 280 nm, the protein concentration of each fraction was monitored by the method of Lowry *et al.* (11). Eighty five percent of the insulin-binding capacity (receptors) and 0.85% of the protein of the soluble membrane fraction was adsorbed on a Con A Sepharose column, and 64% of the receptors and 0.47% of the protein was eluted with 0.3 M α -methyl-D-mannoside. Materials bound to the Con A Sepharose column after elution with α -methyl-D-mannoside were not released by a subsequent addition of 1 M NaCl solution containing 0.1% Triton X-100. By this step, insulin receptors were concentrated 135-fold.

Gel Filtration by Sepharose 6B

The elution pattern on a Sepharose 6B column of the insulin-binding fraction from the Con A Sepharose procedure is shown in Fig. 3. Insulin degrading activity was eluted together with the main peak of protein and separated from the receptor fraction (data not shown). Insulin receptors were concentrated 8-fold by this step.

Purification by Gel Filtration of Insulin Receptors Bound to Insulin-Dextran

The receptor fraction from the Sepharose 6B gel

Fig. 2 Affinity chromatography with concanavalin A Sepharose of solubilized plasma membrane fraction of human placenta. Solubilized membrane fraction (525 mg of protein) was applied to a concanavalin A Sepharose column, washed with 130 ml of 50 mM Tris-HCl buffer (pH 7.4) 2 mM CaCl_2 , 2 mM MgCl_2 and 0.1% Triton X-100. Crude insulin receptor fraction was eluted with 40 ml of 0.3 M α -methyl-D-mannoside, and 4.6 ml fractions were collected. Each fraction was assayed for specific insulin binding (—x—) and for protein concentration by Lowry's method (—●—).

Fig. 3 Gel filtration of Con A fraction by Sepharose 6B. Con A fraction (2.48 mg protein) was applied to a Sepharose 6B column and eluted with 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 in 2.3 ml fractions. Each fraction was assayed for specific insulin binding (—x—) and for protein concentration (—●—).

Fig. 4 Gel filtration by Sepharose 6B of the receptor-insulin-dextran complex. a: Excess Triton X-100 in Sepharose 6B fraction was removed by adsorption to Biobeads SM-2 at 4°C, and the fraction (282 µg protein) was incubated with 2 mg of insulin-dextran II in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 for 12 hr at 4°C. It was applied to a Sepharose 6B column and eluted with the same buffer in 2.3 ml fractions. Each fraction was assayed for radioactivity of insulin-dextran (—○—), protein concentration (—●—), and specific insulin binding (—x—). Peaks I and II are receptor-insulin-dextran complexes and peak III corresponds to free insulin-dextran. b: Fractions 26–30 (a part of peak II) were pooled, acidified to pH 6.0 with 1 N HCl and applied to a Sepharose 6B column equilibrated with 50 mM Tris-HCl buffer (pH 6.0) containing 0.1% Triton X-100. Each fraction (2.3 ml) was neutralized to pH 7.4 and assayed for radioactivity of insulin-dextran II (—○—), protein concentration (—●—), and specific insulin binding (—x—). The specific insulin binding is now observed as a single peak between peak II and peak III.

filtration was further purified by its binding to soluble insulin-dextran II followed by gel filtration with a Sepharose 6B column. Fig. 4a shows the elution profile of protein, receptors, and radioactivity of insulin-dextran II by this procedure. Peaks I and II correspond to the receptor-insulin-dextran complex and peak III to free insulin-dextran II. Addition of excess native insulin decreased the radioactivity in peak I and peak II, and increased that of peak III. This receptor-insulin-dextran complex was eluted before the major peak of protein. A small amount of receptors unbound to insulin-dextran was eluted after peak II. Insulin-binding activity was masked in the fractions of the receptor-insulin-dextran complex, because the receptor-binding site was occupied by insulin-dextran II. A part of peak II (fractions 26-30) was pooled and acidified to pH 6.0 and gel-filtered again on the same Sepharose 6B column equilibrated at pH 6.0 (Fig. 4b). The receptor-insulin-dextran complex was dissociated in this condition. The major peak of insulin-binding (free receptors) was eluted between small peaks of the receptor-insulin-dextran complex and the free insulin-dextran. Thus, insulin receptors almost free of insulin-dextran were obtained. About 7-fold purification was achieved at this step. Altogether, 8,700-fold purification of insulin receptors was obtained with 2.6% recovery (Table 1). The purification was 59-fold and the recovery was 11% after the Con A Sepharose affinity chromatography.

Estimation of Molecular Weight and Affinity Constants of Insulin Receptors

A Sepharose 6B column was calibrated with aldolase, catalase and apoferritin. The K_d value for insulin receptors was 0.45 in the presence of 0.1% Triton X-100, corresponding to 300,000 as an approximate molecular weight

Fig. 5 Estimation of molecular weight (MW) of purified insulin receptors using Sepharose 6B gel filtration. A Sepharose 6B column was equilibrated with 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, and calibrated with aldolase (MW: 158,000), catalase (MW: 232,000), apoferritin (MW: 440,000), and blue dextran. These protein markers were detected by Lowry's method. Their elution profiles were compared with that of the purified insulin receptors.

(Fig. 5). The molecular weight of insulin-dextran II and the receptor-insulin-dextran complex (peak II) was about 150,000 and 450,000, respectively, as estimated by radioactivity.

At each step of purification, Scatchard analysis was performed and the affinity constants were calculated (3). Affinity constants for both high- and low-affinity receptors did not change signi-

Table 1 Steps of Purification of Insulin Receptors from Plasma Membrane Fraction of Human Placenta

Preparations	Protein (mg)	Specific insulin binding (mol/mg protein)	ratio	Total binding of insulin (mol)	Recovery (%)	K_e (M^{-1})	K_f (M^{-1})
$10^6 \times g$ pellet	1,280	2.9×10^{-13}	1	3.71×10^{-10}	100	1.03×10^{10}	1.45×10^9
Solubilized membrane	525	3.1×10^{-13}	1.08	1.63×10^{-10}	44.6	9.9×10^9	9.2×10^8
Con A fraction	2.48	3.95×10^{-11}	146	9.80×10^{-11}	29.0	1.45×10^{10}	1.36×10^9
Sepharose 6B fraction	0.282	3.25×10^{-10}	1,160	9.17×10^{-11}	26.4	1.33×10^{10}	1.02×10^9
Purified receptors	0.0046	2.08×10^{-9}	8,700	9.57×10^{-12}	3.1	1.06×10^{10}	9.6×10^8

K_e , empty site affinity; K_f , filled site affinity. Scatchard plots were analyzed according to De Meyts and Roth (3).

ificantly during the whole purification process (Table 1).

DISCUSSION

As a means of purification of insulin receptors, solid phase affinity chromatography using insulin-agarose or concanavalin A agarose has been widely employed (1, 2, 8, 12, 13, 20). More recently, an elaborate method of immunoaffinity chromatography was described by Heinrich *et al.* (5). In this method, labeled insulin was first cross-linked covalently with insulin receptors, the solubilized membrane was incubated with anti-insulin serum, and the resultant antibody-insulin-receptor complex was collected by a passage through a Sepharose column coupled with protein A from *Staphylococcus aureus*, which binds IgG selectively. About 2,000–3,000-fold purification of insulin receptors has been achieved by these methods.

Insulin-dextran is a stable, soluble macromolecule retaining specific binding property with insulin receptors (16–19). We found that insulin-dextran had insulin-like activity with regard to glucose oxidation, lipogenesis, antilipolysis, and pyruvate dehydrogenase activation in adipose tissue (16, 17). We explored the possibility that this material could serve as a carrier for the purification of insulin receptors. Such a principle of the use of a soluble biospecific macromolecule was recently applied by Hubert *et al.* (7) for the purification of estrogen receptors from calf uterus. In their method, the estrogen receptors were bound to a dextran-estradiol conjugate and the complex was isolated by gel filtration. The present study confirmed that the same principle can be successfully applied also for the purification of insulin receptors from the placenta. Crude insulin receptor fraction was reacted with insulin-dextran, and the receptor-insulin-dextran complex thus formed was separated by gel filtration, and the receptors were dissociated from insulin-dextran by adjusting the pH to 6.0. This mild acidification was adequate to dissociate the receptors from insulin-dextran. This method permitted 8,700-fold purification of insulin receptors from crude plasma membrane, and about 60-fold purification after the step of concanavalin A Sepharose affinity chromatography. The final specific activity was 2.08×10^{-9} mol per mg protein, which corresponds to 0.6 insulin molecule per receptor molecule, assuming the molecular weight of the receptor as 300,000 daltons. It is not yet elucidated how many native insulin molecules can be actually

bound to one receptor. Therefore, the theoretical purity of the final receptor preparation could not be calculated. It seems possible, however, that insulin-dextran and receptor were bound mostly in a 1:1 ratio and partly in a 1:2 or 2:1 ratio, because there were two peaks of receptor-insulin-dextran complexes of different molecular weight (Fig. 1).

The merits of using a soluble biospecific macromolecule are as follows. First, non-specific contaminating proteins adsorbed to insulin-dextran will be smaller than when working with a column of insulin-agarose, because a smaller absolute amount of biospecific macromolecules is needed to bind insulin receptors by the use of insulin-dextran. Non-biospecific adsorption may interfere with the purification process of bioactive material in work with solid phase affinity columns (14). Second, receptor binding occurs under equilibrium conditions in a soluble state, permitting an easier control of reaction conditions than in solid phase reaction. The receptor bound to insulin-dextran can be subjected to direct gel filtration and easily isolated. We suggest that this principle can be applied for the purification of various bioactive substances as an alternative to conventional affinity chromatography.

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