Tyrosine kinase activity of insulin receptors from an insulin-resistant patient with leprechaunism

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Summary. Defects in insulin receptor function can impair the response of target cells to insulin. Previously we have described an insulin resistant patient (leprechaun/Ark-1) with qualitative abnormalities in insulin binding suggestive of a structural defect in her insulin receptors. In the present work, we have studied the tyrosine kinase activity associated with insulin receptors from cultured Epstein-Barr virus-transformed lymphocytes. In studies of insulin receptors from leprechaun/Ark-1, we observed that both the magnitude and the dose-dependency of insulin's effect to stimulate the tyrosine kinase activity were normal. This suggests that the defect causing this patient's insulin resistance is independent of the receptor-associated tyrosine kinase. In the course of these

studies, we noted that an anti-receptor antiserum (B-d) had a markedly decreased ability to immunoprecipitate insulin receptors from leprechaun/Ark-1. This observation further supports our previous conclusion that the insulin receptor from leprechaun/Ark-1 is abnormal in structure. Moreover, it emphasizes the importance of choosing anti-receptor antisera which are equally effective at immunoprecipitating receptors from both patients and normal subjects when the anti-receptor antisera are employed as reagents in investigations of receptors from insulin-resistant patients.

Key words: Insulin receptor, tyrosine kinase, insulin resistance, anti-receptor antibodies, leprechaunism.

Insulin resistance plays an important role in the pathogenesis of several disease states, including Type 2 (non-insulin-dependent) diabetes mellitus. Recent evidence supports the hypothesis that activation of the insulin receptor-associated tyrosine kinase mediates the biological action of insulin [1–3]. Moreover, several reports have suggested that defects in the receptor-associated tyrosine kinase may cause insulin resistance in some patients [4–8].

Previously we have described a patient with extreme insulin resistance (leprechaun/Ark-1) whose cultured Epstein-Barr virus-transformed lymphocytes possess a normal number of insulin receptors [9–12]. However, the receptors exhibit multiple qualitative abnormalities in insulin binding including increased affinity and decreased sensitivity to changes in temperature and pH, suggesting an abnormality in receptor structure. Moreover, because the patient's receptor bound insulin with increased affinity, the insulin resistance was not caused by a failure to bind insulin, but more likely by a defect in transmembrane signalling. In the present investigations, we have inquired whether the defect in transmembrane signalling might be caused by a defect in the receptor's tyrosine kinase ac-

tivity. Because insulin had a normal effect to stimulate tyrosine kinase activity of receptors from leprechaun/Ark-1, we conclude that the cause of insulin resistance is likely to be independent of kinase activation. In addition, we observed that anti-receptor antiserum (B-d) had a decreased ability to bind to insulin receptors from leprechaun/Ark-1. These data provide further evidence to support the previous conclusion that insulin receptors of leprechaun/Ark-1 have an intrinsic structural abnormality. Moreover, because anti-receptor antibodies have frequently been employed as reagents to study insulin receptors from insulin resistant patients, our studies draw attention to an important methodological pitfall which must be avoided in this type of study.

Subjects and methods

Patients and cell lines

The insulin resistant patient referred to as leprechaun/Ark-1 is a young girl with leprechaunism who has been the subject of several publications [9–15]. She exhibited several of the classical phenotypic features of leprechaunism, including intrauterine growth retardation (weight 2,600 g at birth), extreme insulin resistance, hyperinsulin-

Fig. 1. Affinity labelling of insulin receptors in cultured lymphocytes. Insulin receptors on cultured lymphocytes were affinity-labelled as described in Methods and analyzed by SDS-polyacrylamide gel electrophoresis with (lanes A, B) or without (lanes C, D) prior reduction of disulfide bonds by β -mercaptoethanol. Different percentages of polyacrylamide were used in lanes A-B (7.5%) and lanes C-D (5%). Samples in lanes A and B were immunoprecipitated with α -IR-1 monoclonal antireceptor antibody [20]. Samples in lanes C and D were analyzed by SDS-polyacrylamide gel electrophoresis directly after solubilisation of the cells.

aemia (insulin levels ranging from 125–10,000 μ U/ml) and fasting hypoglycaemia [14]. The mother had normal glucose tolerance but similar qualitative defects in insulin binding to Epstein-Barr virus-transformed lymphocytes. The father had a moderate degree of insulin resistance and hyperinsulinaemia (insulin levels ranging from 90–485 μ U/ml; fasting glucose 3.9 mol/l). There was no history of consanguinity.

The normal subjects employed for these studies, P.L. and Y.G. [16], were chosen as representative because the insulin receptor on their cultured cells had been characterised in great detail in previous studies. Epstein-Barr virus transformed lymphocytes cell lines, established from peripheral blood lymphocytes as described previously, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Bio-Fluid Laboratories Inc, Rockville, Md, USA) [9, 17].

Cross-linking

125 I-Insulin was covalently cross-linked to insulin receptors on intact cells using 0.1 mmol/l disuccinimidyl suberate (Pierce Chemical Co., Rockford, Ill, USA) as described previously [18, 19]. Cells were solubilized in 1% (volume/volume) Triton X-100 and the insoluble material was sedimented by centrifugation (200,000 g for 45 min at 4°C). The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis with or without prior reduction of disulfide bonds with β -mercaptoethanol (2.5% volume/volume). In some cases, the detergent extract was subjected to immunoprecipitation with α -IR-1 monoclonal anti-insulin receptor antibody (an antibody with specificity to immunoprecipitate the insulin receptor with much greater

Fig. 2. Insulin-stimulated phosphorylation of the insulin receptor β -subunit. Partially purified preparations of insulin receptors from the normal subject (lanes C, D, G, H) and the patient (lanes A, B, E, F) were phosphorylated either in the absence (lanes A, C, E, G) or in the presence (lanes B, D, F, H) of 10^{-7} mol/l insulin as described in Methods. Some samples of 32 P-phosphoprotein were subjected to immunoprecipitation with B-d antireceptor antiserum (1:100 dilution) (lanes E–H). With other samples from the same experiment, immunoprecipitation was omitted (lanes A–D). After reduction of disulfide bonds by β -mercaptoethanol, the phosphoproteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. In this experiment, receptor concentrations were adjusted to give 125 I-insulin binding activity of approximately 0.24 (ratio of bound/free radioligand) assayed at an 125 I-insulin concentration of 0.1 ng/ml for both leprechaun/Ark-1 and the normal subject. Note that the wheat germ agglutinin-agarose purified insulin receptor from leprechaun/Ark-1 has a normal binding affinity [10]

affinity than the IGF-I receptor) [20] prior to preparation for analytical SDS-polyacrylamide gel electrophoresis (Fig. 1, lanes A, B). In some other cases, aliquots of the detergent extract were subjected to immunoprecipitation with anti-receptor antibody B-d [23] (Fig. 4). Immunoprecipitated samples and corresponding supernatants were analyzed by SDS-polyacrylamide gel electrophoresis following reduction of disulfide bonds with β -mercaptoethanol (2.5% volume/volume). The radioactivity associated with the α -subunit of the insulin receptor was determined by γ -counting.

Wheat germ agglutinin-agarose chromatography

Insulin receptors were partially purified by chromatography over wheat germ agglutinin-agarose according to the method of Hedo et al. [21]. Detergent extracts were applied to wheat germ agglutinin-agarose (Vector Laboratories, Burlingame, Calif, USA) (1 ml packed gel volume). The "flow-through" was reappplied to the column two additional times. The column was then washed with 80 volumes of wash buffer (Hepes, 50 mmol/l, pH. 7.8; NaCl, 150 mmol/l; Triton X-100, 0.1% volume/volume). Thereafter the receptors were eluted with six aliquots (1 ml) of N-acetyl-glucosamine (0.3 mol/l) dis-

Fig. 3. ^{32}P -incorporation into the β -subunit of insulin receptors with and without immunoprecipitation. The autoradiogram from Fig. 2 was subjected to scanning densitometry to quantitate the incorporation of ^{32}P into the insulin receptor β -subunit. With both the non-immunoprecipitated and the immunoprecipitated samples, the ^{32}P associated with the β -subunit of the insulin-stimulated normal receptor (lanes D and H, respectively) was assigned a value of 100 arbitrary units

Fig. 4. Immunoprecipitation of affinity-labelled insulin receptor by antibody B-d. Insulin receptors on cultured lymphocytes from leprechaun/Ark-1 and the normal subject were affinity labelled as described in Methods. The detergent extracts were immunoprecipitated using increasing amounts of IgG from anti-receptor antibody B-d. The IgG fraction from the serum was obtained by affinity chromatography over protein A-sepharose (Pharmacia, Upsalia, Sweden). The IgG concentration was determined spectrophotometrically [28]. Immunoprecipitated samples and corresponding supernatants were analyzed by SDS polyacrylamide electrophoresis after reduction of disulfide bonds by β -mercaptoethanol. Following autoradiography of the dried gel, the bands corresponding to the α -subunits were excised (upper panel) and the associated radioactivity was determined by γ -counting. The results in the lower panel are expressed as the percentage of the radioactivity present in the α -subunit of the immunoprecipitated samples as a function of the total radioactivity present in the α -subunit of the immunoprecipitated samples and the supernatants

solved in wash buffer. To determine whether receptors from leprechaun/Ark-1 interact normally with wheat germ agglutinin-agarose, we carried out wheat germ agglutinin affinity chromatography with affinity-labeled receptors from leprechaun/Ark-1 and a normal subject. There was no significant difference in the recovery of affinity-labeled insulin receptors from leprechaun/Ark-1 ($53 \pm 1\%$) and the normal subject ($56 \pm 6\%$). Recoveries were calculated by quantitation of the radioactivity associated with the receptor α -subunit before and after chromatography over wheat germ agglutinin-agarose. The data are derived from three separate experiments, each with triplicate determinations.

Phosphorylation

Plasma membranes, prepared from 2×10^9 cells, were solubilised in 1% (volume/volume) Triton X-100 as described previously [10, 22]. The following protease inhibitors were added during the solubilisation procedure: phenylmethylsulfonyl fluoride (2 mmol/l), aprotinin (0.1 mg/ml), leupeptin (0.1 mg/ml), pepstatin A (0.1 mg/ml), α_2 macroglobulin (0.5 U/ml). After insoluble material was removed by centrifugation at 200,000 g for 45 min, insulin receptors were partially purified by chromatography over wheat germ agglutinin-agarose (vide supra). ^{125}I -insulin binding activity in the column eluates was measured at 4°C as described previously [10]. Under these conditions, the wheat germ agglutinin-purified insulin receptors from leprechaun/Ark-1 have normal binding affinity [10]. Based on these measurements of ^{125}I -insulin binding, we diluted the preparation of receptors to achieve equal concentrations of insulin receptor binding sites for both leprechaun/Ark-1 and the normal subject. Because there is considerable variation in receptor number even among normal subjects [12], it was necessary to equalise ^{125}I -insulin binding activities (ratio of bound/free radioligand assayed at an ^{125}I -insulin concentration of 0.1 ng/ml) in the extract from cells of leprechaun/Ark-1 and the representative normal subject whose cells were studied simultaneously.

The receptor preparations were incubated either in the absence or in the presence of insulin (as specified in the legends) for 1 h at 24°C (or overnight at 4°C for receptor autophosphorylation at 4°C). The phosphorylation reaction was initiated by the addition of Mn acetate (6.8 $\mu\text{mol/l}$), ATP (6.8 mmol/l), CTP (1.3 mmol/l) and 100 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2000 Ci/mmol). After incubation at 24°C for 20 min, the reaction was stopped by the addition of a "stopping solution" containing ATP (15 mmol/l), EDTA (16 mmol/l), NaF (320 mmol/l), Na pyrophosphate (32 mmol/l) and Na vanadate (3.2 mmol/l). We also studied receptor autophosphorylation at 4°C . The incorporation of ^{32}P was greatly reduced at the lower temperature; the magnitude of the reduction was similar with receptors from both the normal subject as well as leprechaun/Ark-1 (data not shown). Most studies were carried out at 24°C because the greater ^{32}P incorporation at that temperature facilitated the studies. For some experiments (Fig. 2, Lanes E-H), receptors were immunoprecipitated with anti-receptor antiserum B-d [23] prior to analysis by SDS-polyacrylamide gel electrophoresis. In other experiments, after addition of 5X concentrated Laemmli buffer [24], the samples were boiled and analyzed by SDS-polyacrylamide gel electrophoresis.

Tyrosine kinase activity

Tyrosine kinase activity was assayed according to the method of Grunberger et al. [28]. The wheat germ agglutinin-purified receptor preparation (20 μl) was incubated in the absence or the presence of insulin (10^{-9} to 10^{-6} mmol/l) in a total volume of 30 μl in a buffer containing Hepes (50 mmol/l; pH 7.6) at 24°C for 1 h (Fig. 6). The reaction was initiated by addition of 40 μl of the synthetic copolymer of glutamic acid plus tyrosine in a 4:1 ratio (poly glu:tyr) (Sigma Corp; St. Louis, Mo, USA) (4 mg/ml), Mg Cl_2 (10 mmol/l), ATP (100 $\mu\text{mol/l}$), and 15 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2000 Ci/mmol) in a total volume of 90 μl . The reaction was stopped after 30 min by spotting 70 μl of reaction mixture on filter paper (Whatman 3 MM) and immersing the paper in a solution of trichloroacetic acid 10% (weight/volume) and sodium pyrophosphate (10 mmol/l).

Fig. 5. Dose-response curves for insulin stimulated phosphorylation of the insulin receptor β -subunit. Solubilized, partially purified insulin receptors from leprechaun/Ark-1 and the normal subject were phosphorylated in the absence or in the presence of insulin (10^{-9} to 10^{-6} mol). The samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The radioactivity incorporated into the β -subunit was determined by excising the corresponding bands in the gel and counting them in a liquid scintillation counter. The results shown represent the mean of two different experiments. The ^{125}I -insulin binding activities expressed as B/F (ratio bound/free radioligand), of the receptor preparations used in the two separate experiments were B/F=0.27 (experiment I) and B/F=0.16 (experiment II) assayed at an ^{125}I -insulin concentration of 0.1 ng/ml

Results

Cross-linking studies

We employed affinity labelling techniques to study the oligomeric structure of the patient's insulin receptors and the molecular weight of the alpha subunit (Fig. 1). When the disulfide bonds in the receptor were reduced with β -mercaptoethanol prior to SDS-polyacrylamide gel electrophoresis, the major band was the alpha subunit with M_r of approximately 130 kDa (Fig. 1, lanes A-B). In the experiment presented in Figure 1, the alpha subunit of receptors from leprechaun/Ark-1 had a slightly larger M_r (approximately 5 kDa) than the normal insulin receptor. Although this difference

Fig. 6. Assay of the tyrosine kinase activity of the insulin receptor. Solubilized wheat germ agglutinin-agarose purified insulin receptors from leprechaun/Ark-1 and the normal subject were incubated in the presence of varying concentration of insulin (from 0 to 10^{-6} mol/l) for 1 h at 24°C. Receptor concentrations were adjusted to give equal ^{125}I -insulin binding activity for both the patient and the normal subject. The phosphorylation of the synthetic copolymer of glutamic acid plus tyrosine in a 4:1 ratio (poly glu:tyr) was analyzed as described in Methods. The results (mean of duplicate determination in two separate experiments) are expressed as pmol of phosphate per mg of poly glu:tyr substrate per unit of insulin binding activity in the course of 30 min

was observed in several independent experiments, it was not entirely reproducible. With Epstein-Barr virus-transformed lymphocytes from the normal subject, ^{125}I -insulin also labeled a minor band with M_r of approximately 250 kDa (Fig. 1, lane A). This band appears to be a part of the insulin receptor, inasmuch as it was immunoprecipitated by a monoclonal anti-receptor antibody. As previously shown by Elsas et al. [15], this minor 250 kDa band was not detected when cells from leprechaun/Ark-1 were affinity labeled (Fig. 1, lane B) although it was reproducibly detected with cells from all other subjects whom we have studied ($n=5$, data not shown).

When the insulin receptor was subjected to SDS-polyacrylamide gel electrophoresis without prior reduction of disulfide bonds, three oligomeric forms with apparent molecular weights of 430 kDa, 340 kDa and 230 kDa were seen (Fig. 1, lanes C-D). The distribution of oligomeric forms is similar for receptors from both the normal subject and leprechaun/Ark-1.

Phosphorylation studies

Because of the possibility that a defect in the receptor-associated tyrosine kinase activity might be associated with insulin resistance, we studied the kinase activity in insulin receptors from leprechaun/Ark-1. Partially purified insulin receptors were incubated with [γ - 32 P]ATP in the presence or the absence of insulin (10^{-7} mol/l). Phosphoproteins were analyzed by SDS-polyacrylamide gel electrophoresis either directly (Fig. 2, lanes A–D) or after immunoprecipitation with antireceptor antiserum B-d (Fig. 2, lanes E–H). In all cases, insulin stimulated phosphorylation of a 95 kDa band corresponding to the β -subunit of the insulin receptor.

In the study where the immunoprecipitation step was omitted, we observed that insulin stimulated the incorporation of 32 P into the β -subunit of receptors from leprechaun/Ark-1 at least as well as with receptors from the normal subject (Fig. 2, lanes A–D; Fig. 3). Moreover, antireceptor antiserum immunoprecipitated the 32 P-labelled β -subunit only about one-third as well with receptors from leprechaun/Ark-1 as with receptors from the normal subject (Fig. 2, lanes E–H; Fig. 3). Studies with affinity-labelled receptors confirmed this result (Fig. 4). The lowest concentration of anti-receptor antibody B-d (50 μ g/ml) immunoprecipitated 10-fold more normal receptors as compared to receptors from leprechaun/Ark-1 (58% versus 6%). However, a 25-fold greater concentration of antibody (1250 μ g/ml) immunoprecipitated 80–90% of both types of insulin receptors. This is consistent with the conclusion that anti-receptor IgG B-d recognises receptors from both normal subjects and leprechaun/Ark-1, but that the receptor from leprechaun/Ark-1 is immunoprecipitated with 10-fold lower avidity.

Dose-response for autophosphorylation

To further characterise the tyrosine kinase activity of the insulin receptor from leprechaun/Ark-1, we performed a dose-response study for the insulin stimulated phosphorylation of the insulin receptor (Fig. 5). Insulin appeared to stimulate the phosphorylation of the insulin receptor from leprechaun/Ark-1 in a similar fashion compared to the simultaneous control. The shape of the dose-response curves for phosphorylation of the β -subunits appears to be very similar for receptors from both the patient and the control. In fact, 32 P incorporation into the β -subunit from leprechaun/Ark-1 appears to be slightly higher than from the control at each point in the curve.

Dose response for tyrosine kinase activity

We also studied the tyrosine kinase activity of the patient's receptors toward an exogenous substrate (Fig. 6). Insulin receptors from leprechaun/Ark-1 appeared to phosphorylate poly glu:tyr (4:1) substrate normally. The dose-response relationship of insulin-stimulated

tyrosine kinase activity appears very similar with receptors from both leprechaun/Ark-1 and the normal subject.

Discussion

Receptor-associated tyrosine kinase

Recent evidence supports the hypothesis that the receptor-associated tyrosine kinase plays an important role in coupling insulin binding to insulin action [1–3]. Several patients have been described in whom insulin resistance is associated with a selective defect in receptor-associated tyrosine kinase activity [4–6]. In previous studies of Epstein-Barr virus-transformed lymphocytes from leprechaun/Ark-1, we have reported that insulin has a normal effect to stimulate receptor phosphorylation in intact cells [25]. However, as emphasised in that publication, we did not distinguish between tyrosine-specific phosphorylation catalyzed directly by the insulin receptor and phosphorylation of serine or threonine residues, presumably catalyzed by another protein kinase [26]. Furthermore, we did not investigate the dose-response relationship for insulin stimulation of receptor phosphorylation. In the present study, we directly characterised the autophosphorylation activity of the solubilized, partially purified insulin receptor of leprechaun/Ark-1 under conditions where phosphorylation is predominantly on tyrosine residues [27]. Moreover, we characterised the tyrosine kinase activity of the patient's insulin receptor using the exogenous substrate poly glu:tyr (4:1 ratio). The autophosphorylation activity of the patient's receptor as well as the tyrosine kinase activity toward an exogenous substrate were stimulated normally by insulin. In particular, both the magnitude and dose-dependency of insulin's effects were normal. Although Epstein-Barr virus-transformed lymphocytes are not a physiological target for insulin action, we utilised these cells as a source of insulin receptors encoded by the patients insulin receptor genes. This should have enabled us to detect primary defects in the patient's insulin receptor which resulted from mutations of the insulin receptor gene. These observations suggest that this patient does not have a primary defect in the activity of the receptor-associated tyrosine kinase. Thus, it is possible that the insulin resistance is caused by a defect which compromises another, previously unrecognised, function of the receptor required for mediating insulin action.

Receptor structure

Our previous investigations demonstrated abnormal binding kinetics for insulin receptors from leprechaun/Ark-1 [9–11]. Therefore, we have inferred that the receptors from this patient have an abnormal structure, possibly resulting from a mutation in the structural gene encoding the receptor. In the present work using

antireceptor antibodies, we present immunological evidence consistent with our previous conclusion that the receptor is structurally abnormal, at least at the epitope(s) recognized by anti-receptor antiserum B-d (Figs. 2–4). In addition, some of our experiments have suggested that the affinity-labelled α -subunit from receptors of leprechaun/Ark-1 may have slightly decreased mobility in SDS-polyacrylamide gel electrophoresis (Fig. 1). This would also provide direct evidence of a structural abnormality in the α -subunit of this patient's receptor. However, possibly because of limitations in the ability of SDS-polyacrylamide gel electrophoresis to resolve small differences in molecular weight, this observation was not completely reproducible. Finally, our studies with Epstein-Barr virus-transformed lymphocytes confirmed previous observations of Elsas et al. [15] demonstrating an abnormal pattern when insulin receptors in the patient's fibroblasts were covalently cross-linked with disuccinimidyl suberate. With normal receptors, a minor band ($M_r=250$ kDa) is observed which is thought to represent an α - α dimer [15]. This $M_r=250$ kDa band was not detected in cross-linking studies with receptors from leprechaun/Ark-1. This observation, suggesting a defect in the interactions between neighboring α -subunits, is compatible with our previous studies demonstrating defects in the cooperative interactions between insulin binding sites [11].

Heterogeneity of receptors

Previously, we and others have suggested that this patient has inherited two different defective alleles causing insulin resistance, one from each parent [12, 15]. This heterozygosity may give rise to two pools of insulin receptors, the existence of which potentially complicates our studies. Fortunately, the observation that the recovery of receptors from leprechaun/Ark-1 during chromatography over wheat germ agglutinin-agarose is normal makes it unlikely that the chromatographic procedure selectively enriches one pool of receptors. Nevertheless, the presence of two different pools of receptors might explain the results of our studies in which receptors were immunoprecipitated with anti-receptor antibodies. However, our data do not distinguish whether the decreased efficiency of immunoprecipitation of insulin receptors from leprechaun/Ark-1 results from a decrease in affinity to bind all of the patient's receptors or a selective decrease in affinity toward only one of the two pools.

The β -subunit of the IGF-I receptor has M_r of approximately 95 kDa, and is bound with relatively low affinity by anti-insulin receptor antibodies. Thus, it is important to consider whether the ^{32}P -labelled bands are derived from receptors for insulin or IGF-I. As previously reported, we have not detected receptors for IGF-I in Epstein-Barr virus-transformed lymphocytes [17]. Moreover, the dose-response relationship for insu-

lin's effect to stimulate phosphorylation of both normal and leprechaun/Ark-1 receptors is characteristic of a receptor for insulin rather than IGF-I. Therefore, it is likely that the ^{32}P -labelled bands with $M_r=95$ kDa correspond to the β -subunit of the insulin receptor.

Anti-receptor antibodies

Anti-receptor antibodies have been employed in many studies of the tyrosine kinase activity of insulin receptors from insulin resistant patients [4–6, 25]. In our study, we have identified a potential methodological pitfall in this approach. If the insulin resistant patient has structurally abnormal receptors, this abnormality may impair the ability of the antibodies to immunoprecipitate ^{32}P -labelled receptors. Thus, if anti-receptor antibodies are to be used, it is important to employ sufficient quantities of an appropriate anti-receptor antibody to quantitatively immunoprecipitate both the abnormal receptor as well as the normal receptor.

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