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# The endogenous functional turkey erythrocyte and rat liver insulin receptor is an $\alpha_2\beta_2$ heterotetrameric complex

Judith L. TREADWAY,*‡ Brian D. MORRISON,* John A. WEMMIE,* Ignacio FRIAS,† Timothy O'HARE,† Paul F. PILCH† and Jeffrey E. PESSIN*

*Department of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242, and †Department of Biochemistry, Boston University, Boston, MA 01605, U.S.A.

Previous studies have indicated that turkey erythrocyte and rat liver membranes contain endogenous $\alpha\beta$ heterodimeric insulin receptors in addition to the disulphide-linked $\alpha_2\beta_2$ heterotetrameric complexes characteristic of most cell types. We utilized $^{125}$I-insulin affinity cross-linking to examine the structural properties of insulin receptors from rat liver and turkey erythrocyte membranes prepared in the absence and presence of sulphydryl alkylating agents. Rat liver membranes prepared in the absence of sulphydryl alkylating agents displayed specific labelling of $M_r$ 400000 and 200000 bands, corresponding to the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes respectively. In contrast, affinity cross-linking of membranes prepared with iodoacetamide (IAN) or *N*-ethylmaleimide identified predominantly the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex. Similarly, affinity cross-linking and solubilization of intact turkey erythrocytes in the presence of IAN resulted in exclusive labelling of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex, whereas in the absence of IAN both $\alpha_2\beta_2$ and $\alpha\beta$ species were observed. Turkey erythrocyte $\alpha_2\beta_2$ heterotetrameric insulin receptors from IAN-protected membranes displayed a 3–4-fold stimulation of $\beta$ subunit autophosphorylation and substrate phosphorylation by insulin, equivalent to that observed in intact human placenta insulin receptors. Turkey erythrocyte $\alpha\beta$ heterodimeric insulin receptors, prepared by defined pH/dithiothreitol treatment of IAN-protected membranes, were also fully competent in insulin-stimulated protein kinase activity compared with $\alpha\beta$ heterodimeric human placenta receptors. In contrast, endogenous turkey erythrocyte $\alpha\beta$ heterodimeric insulin receptors displayed basal protein kinase activity which was insulin-insensitive. These data indicate that native turkey erythrocyte and rat liver insulin receptors are structurally and functionally similar to $\alpha_2\beta_2$ heterotetrameric human placenta insulin receptors. The $\alpha\beta$ heterodimeric insulin receptors previously identified in these tissues most likely resulted from disulphide bond reduction and denaturation of the $\alpha_2\beta_2$ holoreceptor complexes during membrane preparation.

## INTRODUCTION

In most tissues and species examined, the mature insulin holoreceptor is minimally composed of two identical $\alpha$ subunits ($M_r$ 135000) and two identical $\beta$ subunits ($M_r$ 95000) which are disulphide-linked into an $\alpha_2\beta_2$ heterotetrameric complex (for reviews, see Czech, 1985; Kahn, 1985; Pessin *et al.*, 1985; Goldfine, 1987). However, insulin receptors in membrane preparations from avian erythrocytes and from rat and shark liver have been observed to exist in an $M_r$-200000 $\alpha\beta$ heterodimeric state (Aiyer, 1983*a*,*b*; Koch *et al.*, 1986; Haynes *et al.*, 1986; Stuart, 1988). Structural heterogeneity of insulin receptors could have profound effects on transmembrane signalling, activation of the intrinsic protein tyrosine kinase activity and the elicitation of biological responsiveness. For example, $\alpha\beta$ heterodimeric insulin receptors have been reported to be a highly kinase-active species (Fujita-Yamaguchi & Kathuria, 1985), and these smaller receptor subunit forms have been implicated in altered biological responsiveness (Crettaz *et al.*, 1984).

In order to examine the minimal structural requirements of insulin-dependent receptor signalling, we have developed methodology to isolate functional $\alpha\beta$ heterodimeric insulin receptor complexes from native human placenta $\alpha_2\beta_2$ holoreceptors (Boni-Schnetzler *et al.*, 1986; Sweet *et al.*, 1987*a*). Our results indicate that isolated $\alpha\beta$ heterodimeric insulin receptors display markedly decreased binding affinity for insulin (Boni-Schnetzler *et al.*, 1986, 1987; Sweet *et al.*, 1987*b*; Swanson & Pessin, 1989) and lack insulin-stimulated kinase activity when prevented from re-associating to the $\alpha_2\beta_2$ heterotetrameric state (Boni-Schnetzler *et al.*, 1986; Wilden *et al.*, 1989*a*). Since the isolated human placenta $\alpha\beta$ heterodimeric insulin receptor itself does not undergo insulin-stimulated activation of the intrinsic protein tyrosine kinase activity, it would be expected that cells largely characterized by the $\alpha\beta$ heterodimeric insulin receptor subtype would be less responsive and/or less sensitive to insulin. Alternatively, it is possible that $\alpha\beta$ heterodimeric insulin receptors from other species or tissues could function as an insulin-dependent protein kinase. In either case, it is not clear whether $\alpha\beta$ heterodimeric insulin receptor complexes are endogenous to specific tissues such as avian erythrocytes and rat liver *in vivo* or whether they result from a selective reduction of the inter-heterodimeric (Class I) disulphide bonds of the holoreceptor complex during tissue processing *in vitro*. Therefore we have examined the structural and functional properties of insulin receptors isolated from turkey erythrocyte and rat liver membranes and have compared them with those of the well-characterized insulin receptor from human placenta membranes.

## MATERIALS AND METHODS

### Materials

Bovine serum albumin, bacitracin, protease inhibitors, iodoacetamide (IAN), wheat-germ agglutinin (WGA)–agarose and dithiothreitol (DTT) were obtained from Sigma Chemical Co. [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) and Cronex lightening-plus

Abbreviations used: IAN, iodoacetamide; WGA, wheat-germ agglutinin; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; DSS, disuccinimidyl suberate; NEM, *N*-ethylmaleimide.

‡ To whom correspondence should be addressed.

intensifying screens were purchased from New England Nuclear–du Pont Chemical Co. SDS/PAGE reagents, disuccinimidyl suberate (DSS) and XAR-5 film were obtained from Bio-Rad, Pierce and Kodak respectively. $M_r$ standards for electrophoresis were purchased from Bethesda Research Laboratories. Fresh turkey erythrocytes were obtained from the Louis Rich Co., West Liberty, IA, U.S.A. Porcine insulin was kindly provided by Dr. R. Chance, Eli Lilly Co., and $^{125}$I-labelled monoiodoinsulin (375 Ci/g) was prepared as described by Welinda *et al.* (1984) at the Diabetes and Endocrinology Research Center, The University of Iowa.

### Membrane preparation

Membranes were prepared from freshly obtained human placenta by the procedure of Harrison & Itin (1980) and stored (10 mg/ml) at −70 °C in 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.6, 2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 25 mM-benzamidine hydrochloride, 10 μM-leupeptin, 50 trypsin-inhibitory units of aprotinin/ml, 1 mM-1,10-phenanthroline and 1 μM-pepstatin A. Protein concentration was determined by a modification (Wang & Smith, 1975) of the method described by Lowry *et al.* (1951). Turkey erythrocyte membranes were obtained from whole blood (4 litres) mixed with 200000 units of heparin during collection and filtered through several layers of cheesecloth. Plasma and white cells were removed by centrifugation at 2000 ***g*** for 10 min at 4 °C. Erythrocytes were washed six times by resuspension in ice-cold 0.9 % NaCl and centrifugation at 2000 ***g*** for 10 min at 4 °C. The erythrocyte pellet was stored in 0.9 % NaCl at 4 °C for 16 h, then cells were lysed in hypo-osmotic lysis buffer consisting of 8.5 mM-Tris/HCl, pH 7.8, 3 mM-NaCl, 1 mM-dextrose, 2 mM-$MgCl_2$, 70 μM-PMSF and 2 μM-leupeptin. The lysate was repeatedly centrifuged at 3000 ***g*** at 4 °C and resuspended in lysis buffer until a haemoglobin-free supernatant was obtained. The pellets were resuspended in lysis buffer and homogenized in a Dounce homogenizer (25 strokes) using a Wheaton pestle A. The dounced homogenate was centrifuged at 6000 ***g*** for 10 min at 4 °C, and the top layer consisting of the erythrocyte membranes was pooled and recentrifuged at 17500 ***g*** to remove remaining nuclear material. Membranes were suspended in lysis buffer containing 0.2 mM-$MgCl_2$, pelleted at 17500 ***g***, resuspended to approx. 10 mg/ml and stored at −70 °C until use. Membranes from turkey erythrocytes were also prepared in the presence of 1 mM-IAN (IAN-protected) to prevent sulphydryl reduction and possible structural alteration of the insulin receptor during the isolation procedure. In these experiments, 1 mM-IAN was included in the buffers used to wash and lyse the cells and during isolation of the plasma membrane fraction. The final membrane pellet was resuspended as described above in the absence of IAN. Membranes were prepared from freshly obtained livers from male Sprague–Dawley rats in the absence and presence of 20 mM-*N*-ethylmaleimide (NEM) as described (O'Hare & Pilch, 1989).

### Preparation of $\alpha\beta$ heterodimeric insulin receptors

Human placenta and IAN-protected turkey erythrocyte membranes were incubated at 23 °C for 25 min at pH 8.5 by the addition of 1.0 M-Tris, pH 10, followed by incubation with 2.0 mM-DTT for 5 min at 23 °C, as described by Swanson & Pessin (1989). Membrane samples were then diluted with a 10-fold excess of 50 mM-Tris, pH 8.5, 150 mM-NaCl, 2 mM-EDTA and 0.02 % $NaN_3$ (TEN buffer) at 4 °C and centrifuged for 20 min at 48000 ***g***. The membrane pellets were washed and resuspended with TEN buffer, pH 8.5, and solubilized with 1 % Triton X-100 and 10 μM-leupeptin for 30 min at 4 °C, followed by microcentrifugation at 13000 ***g*** for 30 min at 4 °C.

### WGA affinity chromatography

The solubilized membrane samples were applied to 0.5 ml of WGA–agarose and mixed at 4 °C for 3–4 h. The resin was extensively washed with WGA equilibration buffer (50 mM-Hepes, pH 7.4, 0.02 % $NaN_3$, 10 mM-$MgCl_2$ and 0.1 % Triton X-100), and receptors were eluted with 0.3 M-*N*-acetylglucosamine in 50 mM-Tris, pH 8.0, 0.02 % $NaN_3$ and 0.05 % Triton X-100.

### Sucrose gradient velocity sedimentation

Insulin receptors were resolved by sucrose gradient velocity sedimentation (Boni-Schnetzler *et al.*, 1986) using linear gradients of 5–20 % (w/v) sucrose in 50 mM-Tris/HCl, pH 7.6, 0.1 % Triton X-100 and 0.02 % $NaN_3$ (11.5 ml) layered on a 0.5 ml 20 % sucrose cushion. WGA–agarose eluates (200 μl) were loaded on to the gradient and insulin receptor complexes were resolved by centrifugation at 100000 ***g*** for 19 h at 4 °C. Fractions (250 μl) were collected at a rate of 0.4 ml/min by puncturing the bottom of the tubes with an 18 gauge needle.

### Insulin binding

Sucrose gradient fractions were incubated with 0.25 nM-$^{125}$I-insulin for 16 h at 4 °C in a final volume of 0.2 ml in KRH buffer (50 mM-Hepes, pH 7.6, 130 mM-KCl, 1.3 mM-$CaCl_2$ and 1.3 mM-$MgSO_4$) plus 0.1 % BSA. Free $^{125}$I-insulin was separated from the bound hormone by the addition of 0.5 ml of 0.1 % bovine $\gamma$-globulin and 0.5 ml of 25 % poly(ethylene glycol) at 4 °C, followed by microcentrifugation at 13000 ***g*** for 10 min. The pellets were washed with 1.0 ml of 10 % poly(ethylene glycol). Non-specific binding was determined in the presence of 1 μM unlabelled insulin.

### $^{125}$I-Insulin affinity cross-linking

$^{125}$I-Insulin affinity cross-linking was performed using freshly obtained intact turkey erythrocytes following extensive washing with phosphate-buffered saline (PBS; 5 mM-$Na_2HPO_4$, 150 mM-NaCl, pH 7.4). Erythrocytes (12 ml) were incubated for 30 min at 23 °C in the absence or presence of 10 mM-IAN in KRH buffer plus 0.1 % BSA and 0.01 % bacitracin. The cells were then incubated for 16 h with $^{125}$I-insulin (0.25 nM) at 4 °C before the addition of 0.1 mM-DSS for 5 min. The reaction was terminated by the addition of 2.0 ml of 1 M-Tris, pH 10, for 15 min at 23 °C. Non-specific $^{125}$I-insulin affinity cross-linking was determined by inclusion of 1 μM unlabelled insulin in the initial preincubation. The samples were centrifuged at 3000 ***g*** and the cell pellet was washed three times in PBS in the presence or absence of 1 mM-IAN. The cell pellet was then solubilized for 30 min at 4 °C in 2 vol. of 50 mM-Tris/HCl pH 7.4, 150 mM-NaCl, 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS (RIPA buffer) containing 1 μM-pepstatin A, 10 μM-leupeptin, and 1 mM-PMSF. Samples were centrifuged at 100000 ***g*** for 30 min at 4 °C and insulin receptors were partially purified by WGA–agarose affinity chromatography. The WGA–agarose eluates were mixed with Laemmli (1970) sample buffer (50 mM Tris/HCl, pH 6.9, 10 % glycerol, 0.05 % Bromophenol Blue and 1 % SDS) and resolved by SDS/PAGE under non-reducing conditions.

$^{125}$I-Insulin affinity cross-linking to membranes and solubilized receptors was performed as described above, except that reactions were terminated by the addition of Laemmli sample buffer.

### Autophosphorylation and substrate phosphorylation of soluble insulin receptors

WGA–agarose eluates (50 μl) were preincubated for 1 h at 23 °C in the absence or presence of 100 nM-insulin and/or 10 mM-IAN, as indicated in the individual Figure legends, plus 50 mM-Hepes, pH 7.8, 2 mM-$MnCl_2$ and 10 mM-$MgCl_2$. Autophosphorylation was initiated by the addition of [$\gamma$-$^{32}$P]ATP (100 μM,

3 $\mu$Ci/nmol) and terminated after 5 min with Laemmli sample buffer. Exogenous substrate phosphorylation was determined by the addition of 2 mg of Glu:Tyr (4:1) synthetic polymer/ml in the phosphorylation reaction and termination by trichloroacetic acid precipitation on to Whatman 3MM paper.

## SDS/PAGE

Insulin affinity-cross-linked and [$\gamma$-$^{32}$P]ATP-autophosphorylated samples were run on non-reducing 3–10% linear gradient polyacrylamide gels in the presence of SDS according to the procedure of Laemmli (1970). Insulin receptor samples were mixed with Laemmli sample buffer and applied to the 3–10% gels without heating. The gels were stained with Coomassie Brilliant Blue R, dried and autoradiographed with Kodak XAR-5 film using Cronex lightening-plus intensifying screens.

## RESULTS

### $^{125}$I-Insulin affinity cross-linking to membranes

Insulin receptors from turkey erythrocyte and rat liver membranes were structurally analysed by $^{125}$I-insulin affinity cross-linking and compared with the well-characterized insulin receptors from human placenta membranes. Where indicated, the sulphydryl alkylating agent IAN was included in the cross-linking assay to prevent insulin-induced alterations in the association state of any endogenous $\alpha\beta$ heterodimeric insulin receptors present (Wilden *et al.*, 1989*b*). $^{125}$I-Insulin affinity cross-linking of human placenta membranes resulted in the specific labelling of the $M_r$ 400000 $\alpha_2\beta_2$ heterotetrameric insulin receptor species (Fig. 1*a*, lanes 1 and 2). $^{125}$I-Insulin affinity cross-linking to the $\alpha\beta$ heterodimeric human placenta insulin receptor, prepared by treatment of control placenta membranes under defined pH/DTT conditions, demonstrated the specific labelling of both the $M_r$ 400000 and 200000 insulin receptor bands (Fig. 1*a*, lanes 4 and 5). However, incubation of the pH/DTT-treated placenta membranes with 1 mM-IAN resulted in the exclusive $^{125}$I-insulin affinity labelling of the $M_r$ 200000 $\alpha\beta$ heterodimeric insulin receptor complex (Fig. 1*a*, lane 6). These results are consistent with our previous observations that IAN specifically inhibits an insulin-induced covalent association of $\alpha\beta$ heterodimeric insulin receptors into a disulphide-linked $\alpha_2\beta_2$ heterotetrameric state (Wilden *et al.*, 1989*b*). In contrast, pretreatment of the control human placenta membranes with IAN prior to $^{125}$I-insulin cross-linking had no effect on the existing native $\alpha_2\beta_2$ heterotetrameric complex (Fig. 1, lane 3).

Turkey erythrocyte membranes were prepared from intact cells under control (absence of IAN) or IAN-protected conditions to determine the effect of sulphydryl alkylation during the membrane preparation procedure on insulin receptor structure. Membranes prepared in the presence of IAN were resuspended in IAN-free buffer prior to analyses. $^{125}$I-Insulin affinity cross-linking of the IAN-protected turkey erythrocyte membranes specifically identified the $M_r$ 400000 $\alpha_2\beta_2$ heterotetrameric insulin receptor complex (Fig. 1*b*, lanes 1 and 2). $^{125}$I-Insulin cross-linking of control turkey erythrocyte membranes prepared in the absence of IAN demonstrated the specific labelling of both the $M_r$ 400000 $\alpha_2\beta_2$ heterotetrameric and the $M_r$ 200000 $\alpha\beta$ heterodimeric insulin receptor complexes (Fig. 1*b*, lanes 4 and 5). Furthermore, the addition of IAN during $^{125}$I-insulin affinity cross-linking of control turkey erythrocyte membranes did not significantly alter the relative proportions of labelled $M_r$-400000 and -200000 insulin receptor species (Fig. 1*b*, lane 6). This is in direct contrast with human placenta $\alpha\beta$ heterodimeric insulin receptors prepared by defined pH/DTT treatment (Fig. 1*a*, lanes 4 and 6). Taken together, these data suggest that the $\alpha\beta$ heterodimeric insulin receptor complexes in control turkey erythrocyte membranes resulted from reduction of the $\alpha_2\beta_2$ heterotetrameric complex during the membrane preparation procedure,

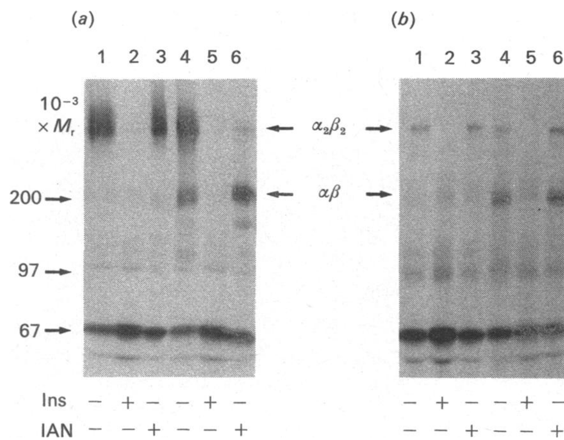


**Fig. 1. $^{125}$I-Insulin affinity cross-linking to human placenta and turkey erythrocyte membranes**

(*a*) Human placenta membrane (HPM) samples were incubated at pH 8.5 in the absence (lanes 1–3) or presence (lanes 4–6) of 2 mM-DTT at 23 °C for 5 min. (*b*) Control (lanes 4–6) and IAN-protected (lanes 1–3) turkey erythrocyte membrane (TEM) samples were isolated from intact cells as described in the Materials and methods section. For cross-linking analysis, the HPM and TEM samples were preincubated in the absence (lanes 1, 2, 4 and 5) or presence (lanes 3 and 6) of 10 mM-IAN plus 1 $\mu$M unlabelled insulin (Ins) (lanes 2 and 5) at 23 °C for 30 min. The samples (100 $\mu$g) were incubated with $^{125}$I-insulin (0.25 nM) for 16 h at 4 °C before DSS cross-linking as described in the Materials and methods section. The samples were then subjected to non-reducing SDS/PAGE (3–10% gels) and autoradiography.

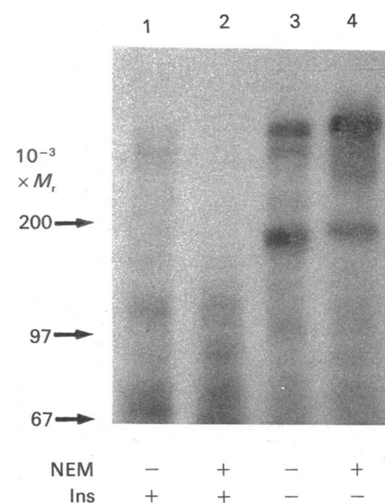


**Fig. 2. $^{125}$I-Insulin affinity cross-linking to insulin receptors from rat liver membranes**

Membranes were isolated from intact rat liver in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 20 mM-NEM. Membranes were then solubilized with 1% Triton X-100 and insulin receptors were partially purified by WGA–agarose affinity chromatography as described in the Materials and methods section. WGA–agarose eluates (50 $\mu$g) were incubated with 0.25 nM-$^{125}$I-insulin for 16 h at 4 °C in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 1 $\mu$M unlabelled insulin (Ins). The samples were affinity cross-linked with DSS, subjected to non-reducing SDS/PAGE (3–10% gels) and autoradiography.

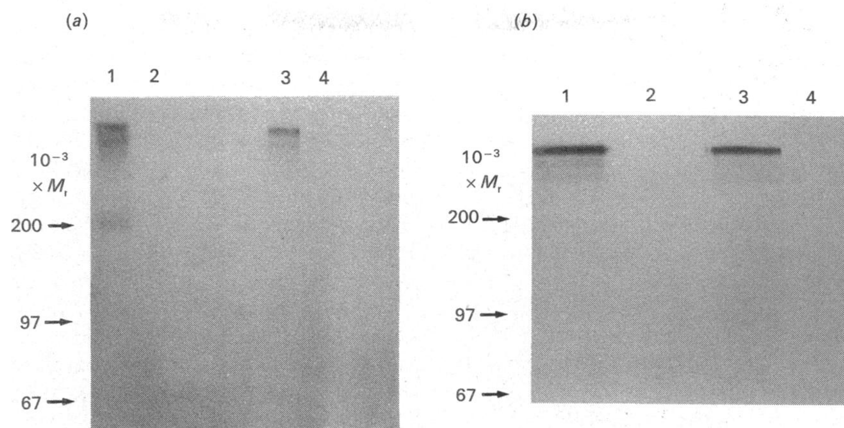


**Fig. 3. $^{125}$I-Insulin affinity cross-linking to intact turkey erythrocytes**

Turkey erythrocytes were preincubated for 30 min at 23 °C in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 mM-IAN plus 1 $\mu$M unlabelled insulin (lanes 2 and 4). $^{125}$I-Insulin (0.25 nM) was then added and the cells were incubated for an additional 16 h at 4 °C before DSS affinity cross-linking. The cells were solubilized in the absence (*a*) or presence (*b*) of 10 mM-IAN in RIPA buffer and then partially purified by WGA–agarose affinity chromatography. The WGA–agarose eluates were subjected to non-reducing SDS/PAGE (3–10% gels) and autoradiography.

and did not reflect the presence of $\alpha\beta$ heterodimeric insulin receptors in the intact turkey erythrocyte.

Rat liver membranes were also examined for the presence of endogenous $\alpha\beta$ heterodimeric insulin receptors. $^{125}$I-Insulin affinity cross-linking of control rat liver membranes demonstrated specific labelling of both $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes (Fig. 2, lanes 1 and 3). However, rat liver membranes prepared with the sulphydryl alkylating agent NEM displayed a markedly decreased number of $M_r$-200000 $\alpha\beta$ heterodimeric insulin receptors, while the proportion of $M_r$-400000 $\alpha_2\beta_2$ heterotetrameric insulin receptors increased concomitantly (Fig. 2, lane 4). IAN and oxidized glutathione were also effective in decreasing and/or preventing the generation of $\alpha\beta$ heterodimeric rat liver insulin receptor complexes during the membrane isolation procedure (results not shown).

### $^{125}$I-Insulin affinity cross-linking to intact cells

To directly assess the insulin receptor association state, intact turkey erythrocytes were analysed by $^{125}$I-insulin affinity cross-linking (Fig. 3). $^{125}$I-Insulin cross-linking of control turkey erythrocytes that were not treated with IAN resulted in the predominant labelling of the $M_r$-400000 $\alpha_2\beta_2$ heterotetrameric insulin receptor, and to a lesser degree of the $M_r$-200000 $\alpha\beta$ heterodimeric complex (Fig. 3*a*, lanes 1 and 2). In contrast, erythrocytes that were affinity cross-linked in the absence (Fig. 3*b*, lanes 1 and 2) or presence (Fig. 3*b*, lanes 3 and 4) of IAN, but which were detergent-solubilized in the presence of IAN, displayed only the $M_r$-400000 insulin receptor species. Since the $M_r$-200000 band was not labelled under these conditions, these results directly demonstrate that the $\alpha\beta$ heterodimers are generated from $\alpha_2\beta_2$ heterotetrameric complexes during the detergent solubilization of intact cells.

### Insulin receptor autophosphorylation

Autophosphorylation of partially purified turkey erythrocyte and human placenta insulin receptors was compared (Figs. 4 and 5). Samples were incubated in the absence or presence of IAN prior to the addition of insulin to determine the effect of receptor association state on the observed kinase activity. WGA–agarose eluates from control human placenta membranes demonstrated

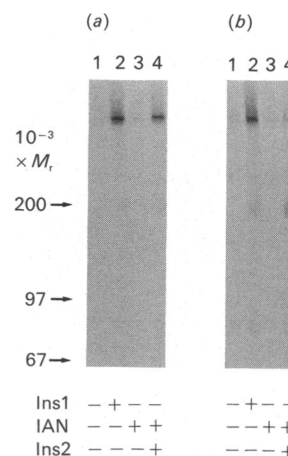


**Fig. 4. Insulin-dependent autophosphorylation of human placenta $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes**

Human placenta membranes (50 mg) were incubated in the absence (*a*) or presence (*b*) of 2 mM-DTT at alkaline pH as described in the legend to Fig. 1. Membranes were then solubilized with 1% Triton X-100 and insulin receptors were partially purified by WGA–agarose affinity chromatography. The WGA–agarose eluates (40 $\mu$l) were preincubated for 1 h at 23 °C in the presence of 100 nM-insulin (Ins1; lane 2) or 10 mM-IAN (lanes 3 and 4). The samples were then incubated in the absence (lanes 1–3) or presence (lane 4) of 100 nM-insulin (Ins2) for an additional 1 h. The autophosphorylation reaction was initiated by the addition of 100 $\mu$M-[$\gamma$-$^{32}$P]ATP (3 $\mu$Ci/nmol) for 5 min and terminated with Laemmli sample buffer. Samples were then subjected to non-reducing SDS/PAGE (3–10% gels) and autoradiography.

a 7-fold insulin-stimulated increase in $\beta$ subunit labelling of the $\alpha_2\beta_2$ heterotetrameric complex (Fig. 4*a*, lanes 1 and 2). Insulin-stimulated autophosphorylation of human placenta $\alpha\beta$ heterodimers obtained by defined pH/DTT treatment resulted in the covalent association to a $^{32}$P-labelled $\alpha_2\beta_2$ heterotetrameric state, with little $^{32}$P labelling of the $\alpha\beta$ heterodimeric species (Fig. 4*b*, lanes 1 and 2). Incubation with 10 mM-IAN prior to the addition of insulin resulted in $\beta$ subunit autophosphorylation predominantly in the $\alpha\beta$ heterodimeric insulin receptor band

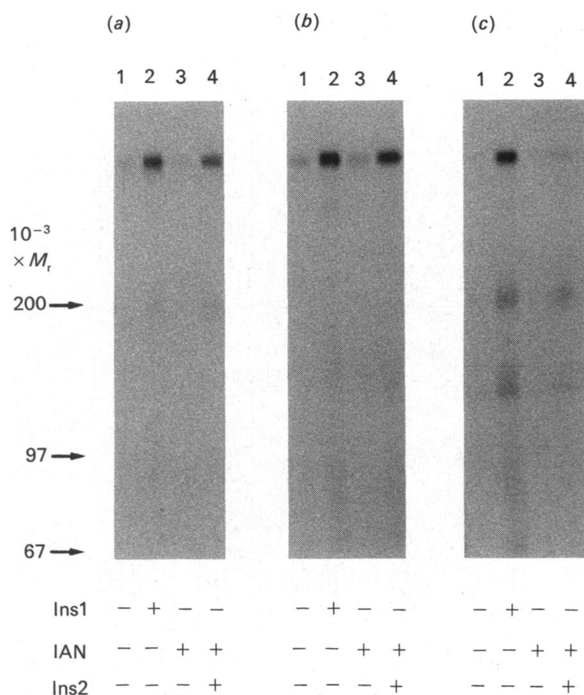


**Fig. 5. Insulin-dependent autophosphorylation of turkey erythrocyte $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes**

Control (*a*) and IAN-protected (*b*,*c*) turkey erythrocyte membranes (50 mg) were isolated as described in the Materials and methods section. The IAN-protected turkey erythrocyte membranes were then subjected to alkaline pH treatment in the absence (*b*) or presence (*c*) of 2 mM-DTT to obtain a defined preparation of $\alpha\beta$ heterodimeric insulin receptors. Membrane samples were solubilized with 1 % Triton X-100 and insulin receptors were partially purified by WGA affinity chromatography. The WGA–agarose eluates (25 μl) were preincubated for 1 h at 23 °C in the presence of 100 nM-insulin (Ins1; lane 2) or 10 mM-IAN (lanes 3 and 4). The samples were then incubated in the absence (lanes 1–3) or presence (lane 4) of 100 nM-insulin (Ins2) for an additional 1 h. The autophosphorylation reaction was initiated by the addition of 100 μM-[γ-$^{32}$P]ATP (3 μCi/nmol) for 5 min and terminated with Laemmli sample buffer. Samples were then subjected to non-reducing SDS/PAGE (3–10 % gels) and autoradiography.

(Fig. 4*b*, lanes 3 and 4). These data are consistent with our previous finding that non-covalent interactions between human placenta insulin receptor $\alpha\beta$ heterodimers support insulin-stimulated intrinsic protein tyrosine kinase activity when covalent association is specifically blocked by IAN (Wilden *et al.*, 1989*b*). As expected, insulin-stimulated autophosphorylation by the control $\alpha_2\beta_2$ heterotetrameric insulin receptors was unaffected by preincubation with 10 mM-IAN (Fig. 4*a*, lanes 3 and 4).

WGA–agarose eluates were prepared from control turkey erythrocyte membranes (Fig. 5*a*), IAN-protected turkey erythrocyte membranes (Fig. 5*b*) and IAN-protected membranes which were alkaline-pH/DTT-treated to reduce the $\alpha_2\beta_2$ heterotetrameric turkey erythrocyte insulin receptors to $\alpha\beta$ heterodimers under the same defined conditions used for human placenta membranes (Fig. 5*c*). The WGA–agarose eluates were incubated with insulin in the absence (lane 2) or the presence (lane 4) of additional IAN prior to autophosphorylation. WGA–agarose eluates from the control turkey erythrocyte membrane preparations demonstrated a 6-fold insulin-stimulated autophosphorylation of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex (Fig. 5*a*, lanes 1 and 2) which was essentially unaltered by pretreatment with 10 mM-IAN in the autophosphorylation assay (Fig. 5*a*, lanes 3 and 4). Although the control turkey erythrocyte preparations contain a substantial amount of $\alpha\beta$ heterodimeric insulin receptor complexes (Fig. 1), only a trace amount of insulin-stimulated autophosphorylation was detected in the $M_r$-200000 insulin receptor band (Fig. 5*a*, lane 2). The partially purified $\alpha_2\beta_2$ heterotetrameric insulin receptors from IAN-protected turkey erythrocyte membranes displayed an approximate 7-fold insulin stimulation of $\beta$ subunit autophosphorylation both in the absence (Fig. 5*b*, lanes 1 and 2) and in the presence (Fig. 5*b*, lanes 3 and 4) of 10 mM-IAN. Autophosphorylation of the WGA eluates from IAN-protected membranes which were alkaline-pH/DTT-treated to generate $\alpha\beta$ heterodimeric insulin receptor complexes resulted in an insulin-induced covalent association into a $^{32}$P-labelled $\alpha_2\beta_2$ heterotetrameric complex (Fig. 5*c*, lanes 1 and 2). Incubation with 10 mM-IAN prior to the addition of insulin significantly inhibited the insulin-induced covalent association into the $\alpha_2\beta_2$ heterotetrameric state (Fig. 5*c*, lanes 3 and 4).

**Sucrose gradient velocity sedimentation**

To further identify the kinase-active insulin receptor species in turkey erythrocyte membranes prepared in the absence of IAN, we isolated the individual $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes by sucrose gradient centrifugation and compared these with the insulin receptor complexes obtained from human placenta and turkey erythrocyte membranes treated under defined pH/DTT conditions (Fig. 6). Sucrose gradient fractions from control human placenta insulin receptors displayed peak insulin binding at fraction 12 corresponding to the $\alpha_2\beta_2$ heterotetrameric complex (Fig. 6*a*). Insulin receptors from alkaline-pH/DTT-treated placenta membranes migrated at fraction 22, corresponding to the $\alpha\beta$ heterodimeric species (Fig. 6*a*). Solubilized control turkey erythrocyte membranes exhibited the presence of both the $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor species (Fig. 6*b*) whereas the IAN-protected membranes displayed only the $\alpha_2\beta_2$ heterotetrameric form of the insulin receptor (Fig. 6*c*). Alkaline-pH/DTT treatment of the IAN-protected turkey erythrocyte membranes resulted in the complete conversion of the $\alpha_2\beta_2$ heterotetrameric insulin receptors into the $\alpha\beta$ heterodimeric state (Fig. 6*c*).

**Insulin receptor substrate phosphorylation**

The sucrose-gradient-isolated insulin receptor species were next examined for insulin-dependent tyrosine-specific exogenous protein kinase activity (Fig. 7). The isolated insulin receptors were incubated with 10 mM-IAN either before (IAN+Ins) or after (Ins+IAN) the addition of 100 nM-insulin to determine the effect of covalent association on substrate protein kinase activity. Insulin stimulation resulted in an approx. 3-fold increase in exogenous substrate kinase activity in both the $\alpha_2\beta_2$ heterotetrameric (□) and $\alpha\beta$ heterodimeric (⊠) insulin receptors from human placenta membranes (Fig. 7*a*, Con versus Ins+IAN). Pretreatment of either the $\alpha_2\beta_2$ heterotetrameric or $\alpha\beta$ heterodimeric human placenta insulin receptors with 10 mM-IAN did not alter the basal (IAN) or insulin-stimulated (IAN+Ins) protein kinase activity.

In agreement with the results obtained for human placenta insulin receptors (Fig. 7*a*), the IAN-protected turkey erythrocyte $\alpha_2\beta_2$ heterotetrameric insulin receptors (□) and the alkaline-pH/DTT-treated $\alpha\beta$ heterodimeric insulin receptors (⊠) also demonstrated an approx. 4-fold insulin stimulation of protein kinase activity (Fig. 7*b*, Con versus Ins+IAN). Furthermore, insulin-stimulated substrate phosphorylation was unaffected by IAN preincubation prior to the kinase assay (Fig. 7*b*, IAN versus IAN+Ins). In contrast, the $\alpha\beta$ heterodimeric insulin receptor

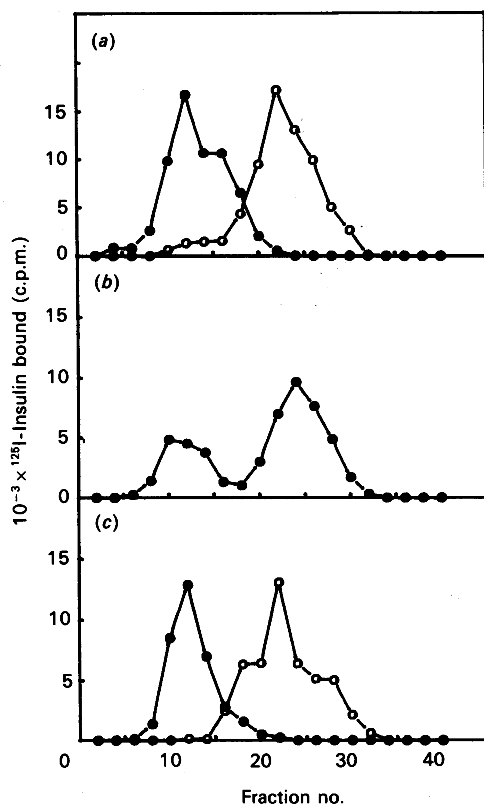


**Fig. 6. Sucrose gradient velocity sedimentation of insulin receptors from human placenta and turkey erythrocyte membranes**

(*a*) Human placenta membranes were incubated at alkaline pH conditions in the absence (●) or presence (○) of 2 mM-DTT, as described in the legend to Fig. 1. The membranes were then solubilized with 1% Triton X-100, and insulin receptors were partially purified by WGA–agarose affinity chromatography. The WGA–agarose eluates (200 μl) were applied to a 12 ml 5–20% linear sucrose gradient and centrifuged at 100000 *g* for 19 h at 4 °C. Fractions (250 μl) were collected by piercing the bottom of the tubes, and aliquots (100 μl) were assayed for $^{125}$I-insulin binding as described in the Materials and methods section. (*b*), (*c*) Control (*b*) and IAN-protected (*c*) turkey erythrocyte membranes were prepared as described in the Materials and methods section. The IAN-protected membranes were further incubated in the absence (●) or presence (○) of 2 mM-DTT under alkaline pH conditions and processed as described for (*a*).

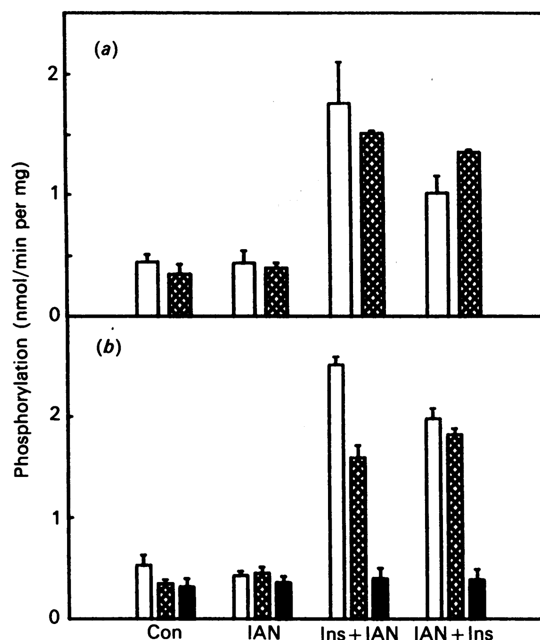


**Fig. 7. Substrate protein kinase activity of isolated human placenta and turkey erythrocyte $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptors**

(*a*) The $\alpha_2\beta_2$ heterotetrameric (□) and $\alpha\beta$ heterodimeric (⊠) insulin receptor complexes obtained from human placenta membranes (HPM) were isolated by sucrose gradient velocity sedimentation as described in the legend to Fig. 6. (*b*) The IAN-protected $\alpha_2\beta_2$ heterotetrameric (□), alkaline-pH/DTT-prepared $\alpha\beta$ heterodimeric (⊠) and endogenously generated $\alpha\beta$ heterodimeric (■) turkey erythrocyte membrane (TEM) insulin receptors were isolated by sucrose gradient velocity sedimentation as described in the legend to Fig. 6. For the substrate phosphorylation assay, HPM and TEM receptor complexes were incubated for 1 h at 23 °C in the absence (Con) or presence of 10 mM-IAN (IAN) or 100 nM-insulin (Ins). Samples were then incubated for an additional 1 h with 10 mM-IAN (+IAN) or 100 nM-insulin (+Ins). The initial rate of Glu:Tyr polymer phosphorylation (2 mg/ml) was determined following the addition of 100 μM-[γ-$^{32}$P]ATP (3 μCi/nmol) as described in the Materials and methods section.

species isolated from control turkey erythrocyte membranes displayed basal protein kinase activity which was completely insulin-insensitive (■). Therefore, in control turkey erythrocyte membrane preparations which contain both $\alpha_2\beta_2$ and $\alpha\beta$ insulin receptor complexes (Fig. 6*b*), only the $\alpha_2\beta_2$ heterotetrameric holoreceptor appears to be an insulin-responsive kinase-active species (Fig. 7*b*).

## DISCUSSION

We have investigated the structure and function of insulin receptors in rat liver and turkey erythrocytes, based on previous observations that these tissues uniquely contain endogenous $\alpha\beta$ heterodimeric insulin receptor complexes (Aiyer, 1983*a*,*b*; Koch *et al.*, 1986; Haynes *et al.*, 1986). Our data demonstrate that both $\alpha\beta$ heterodimeric and $\alpha_2\beta_2$ heterotetrameric insulin receptor species were present in turkey erythrocyte and rat liver membranes prepared in the absence of sulphydryl alkylating agents (Figs. 1 and 2). However, membranes prepared in the presence of sulphydryl alkylating agents displayed only the covalent $\alpha_2\beta_2$ heterotetrameric disulphide-linked insulin receptor complex commonly observed in other tissues, such as human placenta. Furthermore, $^{125}$I-insulin affinity cross-linking of intact turkey erythrocytes demonstrated that the disulphide-linked $\alpha_2\beta_2$ heterotetrameric insulin receptor complex was the only species present (Fig. 3). These data together indicate that $\alpha\beta$ heterodimeric insulin receptor complexes are not endogenous to rat liver or turkey erythrocytes, since the inclusion of IAN in the preparatory buffers completely blocked the appearance of the $\alpha\beta$ heterodimeric species. We therefore conclude that the previous identification of $\alpha\beta$ heterodimeric insulin receptors in rat liver and turkey erythrocytes resulted from an artifactual reduction and dissociation of native $\alpha_2\beta_2$ insulin holoreceptors during the isolation process. Other experimental manipulations have been previously reported to alter the structure of insulin receptors prior to identification, including limited proteolysis during membrane isolation and SDS/heat-dependent intramolecular sulphydryl–disulphide exchange during electrophoresis (Boyle *et al.* 1985; Helmerhorst *et al.*, 1986). As shown in this study, native insulin receptors from rat liver tissue and intact turkey erythrocytes are uniquely susceptible to reduction during membrane isolation and/or detergent solubilization in the absence of

specific sulphydryl alkylating agents. However, the specific intracellular factors released from homogenized rat liver and turkey erythrocytes which result in insulin receptor inter-heterodimeric Class I disulphide bond reduction have not yet been identified. We postulate that such factors are either absent or ineffective in other tissues (e.g. human placenta), since $\alpha\beta$ heterodimeric insulin receptors are not observed when prepared by identical procedures (Aiyer, 1983*a*; Boyle *et al.*, 1985; Fujita-Yamaguchi *et al.*, 1983).

The endogenous $\alpha\beta$ heterodimeric insulin receptor from control turkey erythrocyte membranes is functionally different from $\alpha\beta$ heterodimeric insulin receptors prepared by defined alkaline-pH/DTT treatment of human placental membranes. Human placenta $\alpha\beta$ heterodimeric insulin receptors displayed an insulin-dependent covalent association into an $\alpha_2\beta_2$ heterotetrameric state, as detected both by $^{125}$I-insulin affinity cross-linking (Fig. 1) and by $\beta$ subunit autophosphorylation (Fig. 4), in agreement with previous observations (Boni-Schnetzler *et al.*, 1988; Morrison *et al.*, 1988; Wilden *et al.*, 1989*b*). In contrast, the endogenously generated turkey erythrocyte membrane $\alpha\beta$ heterodimeric insulin receptors did not associate into covalent disulphide-linked $\alpha_2\beta_2$ heterotetrameric complexes (Figs. 1 and 5). In addition, human placenta $\alpha\beta$ heterodimeric insulin receptor complexes prepared under defined alkaline-pH/DTT treatment displayed a 3–5-fold insulin stimulation of $\beta$ subunit autophosphorylation (Fig. 4) and exogenous substrate phosphorylation (Fig. 7), whereas endogenous turkey erythrocyte $\alpha\beta$ heterodimeric insulin receptors displayed basal protein kinase activity which was essentially insulin-insensitive. Thus the $\alpha\beta$ heterodimeric insulin receptors generated by the intrinsic reduction of turkey erythrocyte membranes are qualitatively different from the $\alpha\beta$ heterodimers obtained by defined alkaline-pH/DTT treatment of human placenta membranes. This cannot be accounted for by species/tissue differences alone, since turkey erythrocyte $\alpha\beta$ heterodimeric insulin receptors obtained by alkaline-pH/DTT treatment of IAN-protected $\alpha_2\beta_2$ heterotetramers displayed insulin-stimulated autophosphorylation (Fig. 5) and substrate phosphorylation (Fig. 7) identical to the $\alpha\beta$ heterodimeric human placenta insulin receptor. We conclude that the kinase-inactive state of endogenous $\alpha\beta$ heterodimeric turkey erythrocyte membrane insulin receptors results from reduction and denaturation during the receptor isolation procedure.

In summary, we have demonstrated that both native turkey erythrocyte and rat liver insulin receptors minimally exist in a disulphide-linked $\alpha_2\beta_2$ heterotetrameric state. The presence of lower oligomeric states of the insulin receptors in isolated turkey erythrocyte and rat liver membranes results from an endogenous reduction/dissociation which occurs during the receptor isolation procedure. These endogenously generated $\alpha\beta$ heterodimeric insulin receptor complexes are essentially kinase-inactive and insulin-insensitive. We conclude that native turkey erythrocyte and rat liver insulin receptors share the same $\alpha_2\beta_2$ heterotetrameric structure and mechanism of protein kinase activation as observed for insulin receptors in human placental membranes, as well as other tissues.

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