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The $\alpha\beta$ monomer of the insulin receptor has hormone-responsive tyrosine kinase activity

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Insulin receptors from turkey erythrocyte membranes exist as monomers and dimers when membranes are solubilized with detergent. We examined the ability of monomers and dimers to act as protein kinases to effect both autophosphorylation of the receptor and phosphorylation of an exogenous substrate. After separation by sucrose-density-gradient centrifugation, only receptor dimers show significant basal and insulin-stimulated kinase activity, whereas material at the position of receptor monomers is not active. Partial reduction of the membrane-bound receptors with dithiothreitol, however, produces a receptor monomer containing an α and a β chain which has protein kinase activity similar to that of the original dimers. With rat adipocyte plasma membranes, which in the absence of reducing agents only contain receptor dimers, reduction with dithiothreitol also produces monomers with receptor kinase activity. Receptor monomer hormone-dependent kinase activity is insensitive to receptor concentration and shows stimulation after immobilization on an affinity support.

INTRODUCTION

Biochemical and molecular-biological studies have revealed that the insulin receptor is a membrane-bound glycoprotein composed of two α and two β chains (Ullrich *et al.*, 1985; Massague *et al.*, 1981). Each $\alpha\beta$ complex is synthesized as a single chain and cleaved into α and β chains after insertion into the membrane (Hedo *et al.*, 1983). The α chain, of M_r 135000, is extracellular and has the insulin-binding site (Pilch & Czech, 1979). The β chain, of M_r 90000, contains the transmembrane domain and the intracellular protein tyrosine kinase activity (Roth & Cassell, 1983; Shia & Pilch, 1983; Ullrich *et al.*, 1985). The protein kinase is activated by binding of insulin to the α chain (Kasuga *et al.*, 1982*a,b*, 1983; Jacobs *et al.*, 1983), resulting in autophosphorylation of the β chain (White *et al.*, 1984) and subsequent stimulation of the activity of the kinase on exogenous substrates (Yu & Czech, 1984; Rosen *et al.*, 1983). The α and β chains are held together by disulphide bonds and non-covalent interactions (Czech *et al.*, 1981). The $\alpha\beta$ monomers associate with one another to form the $(\alpha\beta)_2$ insulin receptor (Czech *et al.*, 1981). Since it is possible to convert receptor dimers into monomers by mild treatment with dithiothreitol (Aiyer, 1983*a*; Massague & Czech, 1982), it appears that disulphide bonds are involved in dimer formation. An important question is the relationship of the dimeric structure of the insulin receptor to its function.

It is a popular concept that oligomers of receptor tyrosine kinases are required for activation by hormone (Schlessinger, 1988; Ullrich & Schlessinger, 1990). According to this view, ligand binding causes a conformational change in the extracellular domain of the receptor which induces receptor oligomerization; this leads to activation of the kinase. For disulphide-linked receptor dimers, ligand binding is believed to cause an intra-complex conformational change leading to kinase activation. Indeed, several investigators (Pike *et al.*, 1986; Sweet *et al.*, 1986, 1987; Boni-Schnetzler *et al.*, 1986, 1988; Morrison *et al.*, 1988) have concluded that the $\alpha\beta$ monomers of the insulin receptor do not have hormone-sensitive tyrosine kinase activity.

On the other hand, the group of Rosen (Herrera *et al.*, 1988; Villalba *et al.*, 1989) has expressed and purified the cytoplasmic domain of the human insulin-receptor β subunit, using a baculovirus expression system. The kinase sediments on sucrose density gradients as a monomer, and it undergoes autophosphorylation by an intramolecular process.

In this paper, we show that monomers obtained by reduction of insulin-receptor dimers in the membrane of turkey erythrocytes and rat adipocytes have protein tyrosine kinase activity. Monomers and dimers were separated by sucrose-density-gradient centrifugation. Monomer kinase activity was not dependent on conversion of monomers into dimers, and shows no kinetic effect with dilution. Finally, both receptor monomers and dimers were stimulated to phosphorylate by binding to insulin immobilized to agarose, where monomer association should be minimized.

MATERIALS AND METHODS

Materials

Standard chemicals used were of the highest available grade from Mallinckrodt, Fisher Scientific Co. and Sigma Chemical Co. $Na^{125}I$ and $[\gamma\text{-}^{32}P]ATP$ were obtained from ICN. ^{125}I-Insulin was obtained from DuPont.

Pig insulin was obtained from Eli Lilly. BSA, pig fumarase, chicken lactate dehydrogenase and pig malate dehydrogenase were obtained from Sigma Chemical Co. Histone 2B was obtained from Worthington. Wheat-germ agglutinin (WGA)–agarose was obtained from Vector Laboratories. Agarose-coupled pig insulin was obtained from Sigma.

Buffers and solutions

Triton X-100 was purchased from NEN, and a stock solution of 20% (w/v) was prepared in distilled water. Hepes (Sigma) was used as a buffer for all membrane solutions and suspensions at 50 mM, pH 7.8.

Turkey erythrocyte plasma membranes

Turkey blood was obtained from Raymond's Turkey Farm,

Abbreviations used: WGA, wheat-germ agglutinin; DTT, dithiothreitol.

Methuen, MA, U.S.A. Turkey erythrocyte plasma membranes were prepared by the procedure described by Ginsberg *et al.* (1976). Triton X-100 (final concn. 1–3%, v/v) was added to membranes (protein concn. 12–15 mg/ml), in Hepes buffer at 3 °C. The suspension was ultracentrifuged at 130000 *g* for 45 min, at 3 °C, to yield a supernatant solution containing detergent-solubilized membrane proteins.

Rat adipocyte plasma membranes

Rat adipocyte membranes were prepared by differential centrifugation, by using the method of McKeel & Jarrett (1970) with modifications by Cushman & Wardzala (1980). Membranes were stored in 50 mM-Hepes, pH 7.8, at −70 °C before experiments. Adipocyte membranes were solubilized with Triton X-100 as described above; insulin receptors were further enriched by WGA–agarose purification (Fujita-Yamaguchi *et al.*, 1983).

Preparation of ^{125}I-insulin-labelled detergent extract of membranes

Membranes were labelled and velocity sedimentation was conducted as previously described (Aiyer, 1983*a*,*b*). For velocity sedimentation, marker proteins were identified by SDS/PAGE.

Phosphorylation

Triton-X-100-solubilized turkey erythrocyte and rat adipocyte membranes were diluted, when necessary, to a final Triton concentration of 0.1% and incubated at room temperature for 1 h with rocking in the absence or presence of 100 nM-insulin in 50 mM-Hepes, pH 7.8. Gradient fractions were used directly without dilution. Histone 2B was added to a final concentration of 0.1 mg/ml. Phosphorylation was then initiated by addition of $MgCl_2$, $MnCl_2$ and Na_2ATP to final concentrations of 10 mM, 2 mM and 5 mM respectively. [γ-^{32}P]ATP (7000 mCi/mmol) was added to a final concentration of approx. 1.5 mCi/ml. Reactions were terminated by addition of gel sample buffer in preparation for electrophoresis, or $\frac{1}{4}$ reaction vol. of 250 mM-NaF, 50 mM-$Na_2P_2O_7$ and 25 mM-Na_2EDTA (Stopping Solution) in order to inhibit phosphatase activity, before sucrose-density-gradient sedimentation.

Insulin–agarose-stimulated phosphorylation

Solubilized receptor (100 μl) in 0.1% Triton X-100 was incubated with 10 μl of insulin–agarose for 2 h in 50 mM-Hepes (pH 7.8)/1 mM-EDTA at 3 °C. Insulin–agarose-bound receptor was then quickly pelleted and resuspended twice to remove unbound receptor. Phosphorylation was then effected at 22 °C with standard phosphorylation cocktail in 50 μl volume for 3 min. Phosphorylation was terminated by addition of 50 mM-*N*-ethylmaleimide and gel sample buffer.

Sucrose-density-gradient sedimentation

This was performed as described by Aiyer (1983*a*). To inhibit the action of phosphoprotein phosphatases or isotopic exchange during the sedimentation of phosphorylated receptor, gradient solutions contained 50 mM-NaF, 10 mM-$Na_2P_2O_7$ and 5 mM-Na_2EDTA in addition to buffer.

SDS/PAGE and autoradiography

SDS/PAGE analysis was carried out in the Laemmli (1970) system on gels with an acrylamide gradient (4–8% for receptor alone and 4–20% for receptor and histones). To prevent artifactual reduction of receptor in non-reducing gels, either 10 mM-*N*-ethylmaleimide was added directly to solubilized receptor before addition of gel sample buffer, or 5-fold-concentrated gel sample buffer was made 10 mM with *N*-ethylmaleimide immediately before its addition to solubilized receptor samples. Kodak XAR 5 film was used in conjunction with Cronex Lightning Plus intensifying screens to obtain autoradiographs of the dried gels. Gel slices were then cut, added to a minimum volume of 30% (v/v) H_2O_2, incubated at 85 °C for 15–20 h, and their radioactivity was determined by spectrophotometry in Aquasol.

RESULTS

Protein kinase activity of turkey erythrocyte receptors

Turkey erythrocyte membranes contain insulin-receptor dimers (M_r 350000) and monomers (M_r 220000), with sedimentation coefficients of 10.2 S and 6.6 S respectively, as determined by sucrose-density-gradient centrifugation (Aiyer, 1983*a*,*b*).

To determine whether or not native insulin-receptor monomers have kinase activity, receptor dimers and monomers were isolated by density-gradient centrifugation and the separated peaks were assayed for protein kinase activity on endogenous and exogenous substrates. The results (Fig. 1*a*) show that the principal autophosphorylating and histone-phosphorylating activities are in the dimer peak with a sedimentation coefficient of 10.2 S. On this non-reducing gel the dominant phosphorylated receptor band is seen at M_r 350000. Fig. 1(*b*) shows that the phosphorylating activity of the dimer is insulin-dependent. Fig. 1(*c*), which represents a much longer film exposure, shows that there is very slight phosphorylating activity in the monomer peak, which is also insulin-dependent. The activity of the monomer peak is 40 times smaller than that of the dimer peak per insulin-binding unit.

In order to quantify the kinase activity of receptor dimers and monomers, insulin binding and phosphorylation activity were measured on separated receptor dimers and monomers. Fig. 2 shows the profile for hormone binding and phosphorylation across the gradient. Receptor dimers are the predominant phosphorylated and phosphorylating species; small amounts of both endogenous and exogenous substrate kinase activity can also be observed at the position and M_r of the receptor monomers. Measurement of the amount (mol) of phosphate incorporated per mol of insulin bound gave a ratio of 0.67 for dimers and 0.012 for monomers.

The conclusion is that in the turkey erythrocyte membranes the insulin-receptor dimer is the principal tyrosine kinase activity. It is interesting that in rat liver membranes, which also have insulin-receptor dimers and monomers (Aiyer, 1983*a*), only the dimers have tyrosine kinase activity (G. Velicelebi, personal communication).

Protein kinase activity of reduced insulin receptors

The central question in this work is whether insulin-receptor monomers have protein kinase activity. Since the monomers present in native membranes of turkey erythrocytes are inactive, we asked whether or not the monomers obtained by reduction of active dimers might retain the kinase activity.

Since reduction could activate the kinase activity of the resident receptor monomers in the membrane, we determined the effect of reduction on the properties of the isolated dimers and monomers (Fig. 3). The receptor dimer exhibited insulin-stimulated [^{32}P]phosphate incorporation which was inhibited by dithiothreitol (DTT) in a concentration-dependent manner. Autophosphorylation was not significantly affected by less than 0.5 mM-DTT, but was markedly inhibited at higher concentrations. The binding of insulin to the dimers was increased up to 2-fold by reduction with concentrations of DTT up to 10 mM (results not shown). Monomers, however, were never autophosphorylated, either with or without reducing agents. This

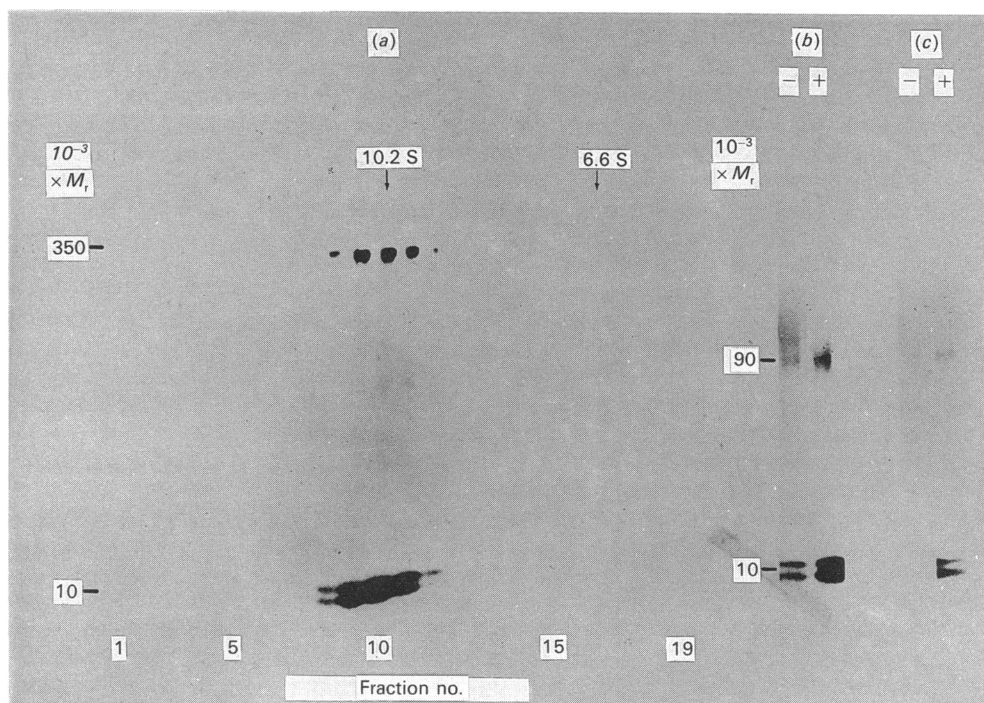

Fig. 1. Phosphorylation of insulin receptors after density-gradient centrifugation

A 200 μl portion of 1% (w/v)-Triton X-100-solubilized turkey erythrocyte plasma membranes was layered on to 5–20% (w/v) sucrose gradients and subjected to ultracentrifugation. Samples (45 μl) of gradient fractions spanning the region containing receptor were incubated with 100 nM-insulin (final concn.) for 1 h at room temperature. After addition of 5 mg of histone 2B, phosphorylation was initiated as described in the Materials and methods section and terminated after 20 min by addition of gel sample buffer. Non-reducing SDS/PAGE was done on 4–20% linear gradient acrylamide gels. Panel (*a*) is the autoradiogram of the gel showing phosphorylation in the presence of insulin. Lanes are shown left to right in order of decreasing sedimentation coefficient. Panels (*b*) and (*c*) show phosphorylation of gradient fractions at 10.2 S and 6.6 S respectively, + and − insulin. These samples were reduced to show phosphorylation of the β-subunit in both dimer and monomer species. Autoradiographic exposure was selected to show the relative phosphorylation of receptor and histone. The exposure time for panel (*c*) is roughly 10 times that for (*b*).

result suggests that exposure of native monomers to DTT will not activate the kinase activity. These results are quantified in Table 1.

Fig. 4 shows the effects of reduction on solubilized receptors. Reduction of phosphorylated receptors by DTT results in the complete conversion of dimers into monomers (lanes 1–9); with 1.5 mM-DTT, half the dimers are converted into monomers (lane 6). Reduction of solubilized receptors with 0.5 mM-, 1.0 mM-, 1.5 mM-, 2 mM- and 5 mM-DTT (lanes A–E) before autophosphorylation causes a decrease in the extent of phosphorylation, even at the lowest concentration of DTT (lane A). At 5.0 mM-DTT (lane E), there is no detectable autophosphorylation. On the other hand, the monomer appears to incorporate low, but detectable, levels of [^{32}P]phosphate at the four lower concentrations of DTT. This result suggests that the monomer can function as a protein kinase.

In order to demonstrate this point clearly, we examined the kinase activity of insulin-receptor monomers obtained by reduction of dimers in the membrane. This procedure was used to minimize the deleterious effects of reduction shown in Fig. 4. Fig. 5 shows that the tyrosine kinase activity of insulin receptors of turkey erythrocyte membranes, as judged by autophosphorylation, is all present at the position of the insulin-receptor dimer. After reduction, however, there is no activity at the dimer position, but a substantial amount of kinase activity is present at the position of the insulin-receptor monomer (6.6 S). Coincident with the loss of kinase activity at the dimer position is a loss of insulin-binding activity at the dimer position (10.2 S) (results not shown). The quantification of this result is shown in Table 2; there is transfer of one half of the autophosphorylating activity and of almost all the histone phosphorylating activity, from the 10.2 S to the 6.6 S position.

The insulin concentration-dependence of the kinase activities of receptor dimers and monomer was compared to estimate the effects of reduction on the properties of the receptor monomer. The results of these experiments, shown in Fig. 6, indicate that both the autophosphorylation activity and the exogenous kinase activity of receptor dimers and monomers have a similar dependence on the insulin concentration with an apparent K_d for insulin of 15 nM. This value is comparable with those obtained for the insulin receptors of 3T3-L1 adipocytes (K_d = 8 nM) (Rosen *et al.*, 1983; Kohanski *et al.*, 1986).

The effect of reduction on the properties of insulin receptors of the rat adipocyte membrane was also studied. In this system, all the insulin receptors are present as dimers. The results shown in Fig. 7 indicate that both autophosphorylating (panel *a*) and histone-phosphorylating (panel *b*) activities are present only at the position of the insulin-receptor dimer before reduction. After reduction with DTT (panel *c*), insulin-dependent histone-phosphorylation activity is found at the monomer position. These results are quantified in Table 3. It is evident that reduction of the adipocyte receptor is accompanied by substantial loss (90–95%) of kinase activities, suggesting a greater sensitivity of this receptor to reduction as compared with the turkey erythrocyte receptor. Although not further investigated, this observation is consistent with the much greater sensitivity of this receptor kinase to bivalent-metal inactivation (E. R. Mortensen, unpublished work).

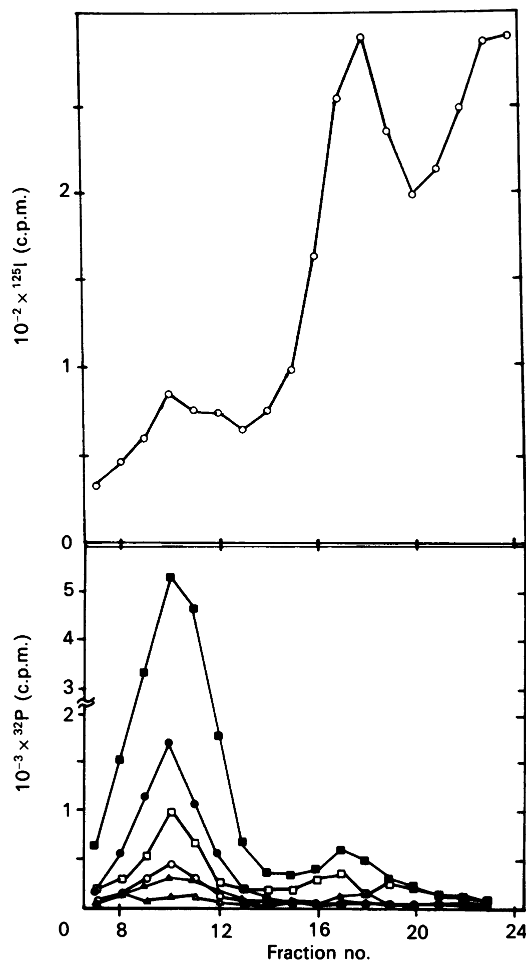

Fig. 2. Insulin binding and kinase activity of insulin-receptor monomers and dimers

Solubilized turkey erythrocyte plasma membrane in 0.5% (w/v) Triton X-100 was incubated with ^{125}I-insulin (50 nM) at room temperature for 1 h. A 200 μl sample was layered on to 5–20% sucrose density gradients and sedimented as described in the Materials and methods section. Samples of fractions were counted for radioactivity with a Beckman 4000 γ-counter. Samples (45 μl) of fractions were incubated with 100 nM-insulin. Histone addition, phosphorylation, SDS/PAGE, autoradiography and quantification of incorporated phosphate were as previously described in the Materials and methods section. Total radioactivity (c.p.m.) in phosphorylated bands at apparent M_r values of 350000 (○, ●), 220000 (△, ▲) and 10000 (□, ■) is indicated (without and with insulin respectively).

Aggregation state of monomers during the kinase reaction

The simplest explanation of these results is that insulin-receptor monomers have tyrosine kinase activity. To rule out the possibility that the monomers might have formed covalently associated dimers in the presence of insulin, the sedimentation properties of receptor monomers from rat adipocyte membranes were determined after autophosphorylation, and after incubation with and without insulin. Fig. 8 shows the analyses of the sucrose density gradients: panel A is the pattern obtained with autophosphorylated monomers; panels B and C show the patterns obtained with the monomer peak after incubation without and with insulin respectively. In all three cases, the only phosphorylated receptor is present at the position of the $\alpha\beta$ monomer (6.6 S), indicating that there is no covalent dimer formed under these conditions.

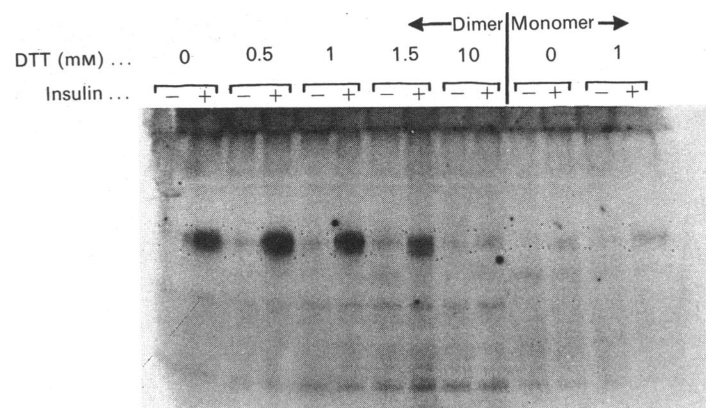

Fig. 3. Phosphorylation of monomers and dimers

Isolated fractions of monomers and dimers were obtained from Triton X-100-solubilized turkey erythrocytes by sucrose-gradient velocity sedimentation (as described in the Materials and methods section). Samples (45 μl) of pooled dimer and monomer fractions were phosphorylated with or without 100 nM-insulin for 20 min in the presence of the indicated concentrations of reducing agent, and analysed as described by reducing SDS/PAGE on 7.5%-acrylamide gels.

Table 1. Effect of reduction on monomer and dimer phosphorylation

The gel bands identified by autoradiography present at the position of monomer and dimer in Fig. 3 were excised, digested and counted for radioactivity as described. Results shown are the incorporated [^{32}P]P$_i$ c.p.m. for minus- and plus-insulin lanes as a function of [DTT]. Also shown is the insulin-dependent c.p.m. [specific = (+insulin)−(−insulin)].

	[DTT] (mM)	^{32}P incorporation (c.p.m.) −Insulin	+Insulin	Specific
Dimer	0	87	350	263
	0.5	169	409	240
	1.0	140	312	172
	1.5	173	221	48
	10.0	118	157	39
Monomer	0	127	126	–
	1.0	120	140	20

There remains the possibility that only non-covalent receptor dimers are active in the kinase reaction. This possibility was examined by measuring the extent of phosphorylation of receptor dimers and monomers of rat adipocyte membranes as a function of receptor concentration. In Fig. 9, lanes A and B, are shown the results of the control and insulin-stimulated phosphorylations of the partially reduced undiluted receptor preparation. The radioactivities of the receptor monomer and dimer bands are reported in Table 4. A 10-fold dilution of the receptor mixture does not affect the fraction of radioactivity in receptor monomer and dimer bands under sub-maximally phosphorylating conditions. This result indicates that the kinase activity of the receptor monomer is not affected by monomer concentration, suggesting that the monomer and not a non-covalent dimer is the active species.

Receptor monomers and dimers obtained from rat adipocytes after reduction with DTT and isolation by sucrose-density-gradient centrifugation were incubated with insulin–agarose in

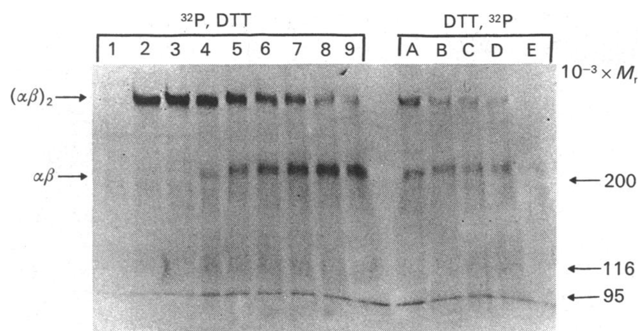

Fig. 4. Analysis of the effect of DTT on receptor kinase before and after phosphorylation

In the left-hand panel, 45 μl samples of solubilized turkey erythrocyte membrane, containing both monomers and dimers, were phosphorylated by the standard protocol, with and without 100 nM-insulin (lanes 1 and 2). This labelled material was subsequently reduced to monomer by a 30 min incubation with 0.1 mM-, 0.5 mM-, 1 mM-, 1.5 mM-, 2 mM-, 5 mM- or 10 mM-DTT (lanes 3–9). In the right-hand panel, solubilized receptor was incubated first with DTT for 30 min and then phosphorylated by the standard protocol, with 100 nM-insulin. The concentrations of DTT were 0.5 mM, 1 mM, 1.5 mM, 2 mM and 5 mM (lanes A–E). Excess *N*-ethylmaleimide was added, and samples were subsequently analysed as described in the Materials and methods section by non-reducing SDS/PAGE on 4%-acrylamide gels.

Table 2. Effect of reduction on the sedimentation properties of turkey erythrocyte receptor kinase activity

As described in Fig. 5, turkey erythrocyte plasma membrane was incubated for 30 min at room temperature with or without 10 mM-DTT. After removal of reductant, membranes were solubilized and velocity-sedimented, and the gradient fractions were phosphorylated and electrophoresed in accordance with standard procedures. Autoradiographically identified phosphorylated bands were excised and counted for radioactivity to a final counting error of 1%. Incorporation of [^{32}P]P$_i$ into receptor monomers and dimers and into histone 2B is shown as a function of the positions of the peaks. Also shown is the ratio of insulin-dependent phosphate incorporation into histone relative to that into receptor β-subunit. Numbers shown are quantitative results of one of four representative experiments.

DTT		^{32}P incorporation (c.p.m.)			Ratio
		−Insulin	+Insulin	Specific	
0	Monomer	26	136	110	
	Histone	167	321	154	1.4
	Dimer	179	1080	901	
	Histone	840	2050	1210	1.3
10 mM	Monomer	321	768	447	
	Histone	701	1911	1210	2.7
	Dimer	21	23		
	Histone	160	86		

[DTT] ... 0 mM 10 mM
10⁻³ × Mr
10.2 S 6.6 S 10.2 S 6.6 S
350
220
10

Fig. 5. Effect of membrane reduction on the sedimentation characteristics of the hormone-dependent kinase activity of turkey erythrocyte membranes

Turkey erythrocyte plasma membrane was incubated with or without 10 mM-DTT for 30 min at room temperature. Membranes were washed, solubilized and velocity-sedimented as described in the Materials and methods section. Samples of fractions were incubated with insulin, phosphorylated, electrophoresed and analysed for incorporated phosphate as described in Fig. 1.

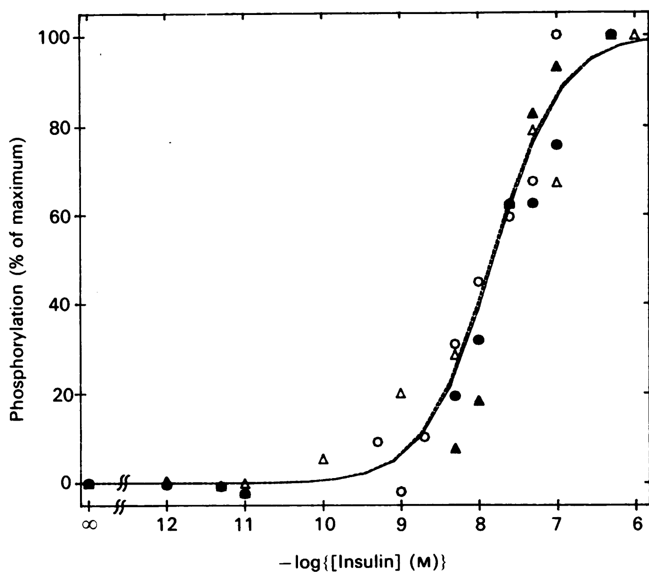

Fig. 6. Dependence of monomer and dimer autophosphorylation and phosphorylation of histone 2B on the insulin concentration

Isolated fractions of receptor monomer and dimer, obtained as in Fig. 5, were incubated with the indicated concentrations of insulin and phosphorylated as described in the Materials and methods section for 20 min. Phosphorylation was terminated by addition of excess *N*-ethylmaleimide and sample buffer. Receptor bands were separated by non-reducing SDS/PAGE on 4%-acrylamide gels, identified by autoradiography, excised and quantified. Percentage of maximal hormone-dependent phosphate incorporation is displayed for autophosphorylation and histone 2B phosphorylation for dimer (○, ●) and monomer (△, ▲) respectively.

order to immobilize the receptors before exposure to the phosphorylation conditions. Lanes C and D of Fig. 9 show that the isolated receptor dimers and monomers, respectively, are capable of autophosphorylation under these conditions. Lane E shows the results obtained with a partially reduced preparation of insulin receptors adsorbed to insulin–agarose. In this case the fraction of radioactivity in receptor monomer and dimer bands is the same as that for the reaction in solution, as is demonstrated in Table 4.

We conclude that the insulin-receptor monomer is active as a kinase as the monomeric species.

DISCUSSION

The data presented here show that, in both turkey erythrocytes and rat adipocytes, the native insulin receptor with protein kinase activity is the $(\alpha\beta)_2$ dimer. After reduction of the disulphide bonds between $\alpha\beta$ monomers, the receptor monomers from turkey erythrocyte (Fig. 5 and Table 2) and rat adipocyte (Fig. 7–9 and Table 3) membranes retain most or part of the kinase activity, respectively.

The simplest explanation of the data is that the $\alpha\beta$ monomer does have protein kinase activity and is competent for autophosphorylation and for phosphorylation of histones.

It has been argued that the $\alpha\beta$ monomer dimerizes under the conditions of the assay for kinase activity, so that in fact the $(\alpha\beta)_2$ dimer is the only species with kinase activity (Boni-Schnetzler *et al.*, 1986; Sweet *et al.*, 1987). This supposition is unlikely for two reasons. In the first place, the results shown in Fig. 5 indicate that the material at the monomer position on the gradient has the size of the $\alpha\beta$ monomer by SDS/PAGE (M_r 220000). Thus, if the dimer had formed during the phosphorylation reaction, the association by non-covalent interactions was not followed by formation of disulphide bonds and stabilization of the dimer. Furthermore, the results shown in Fig.

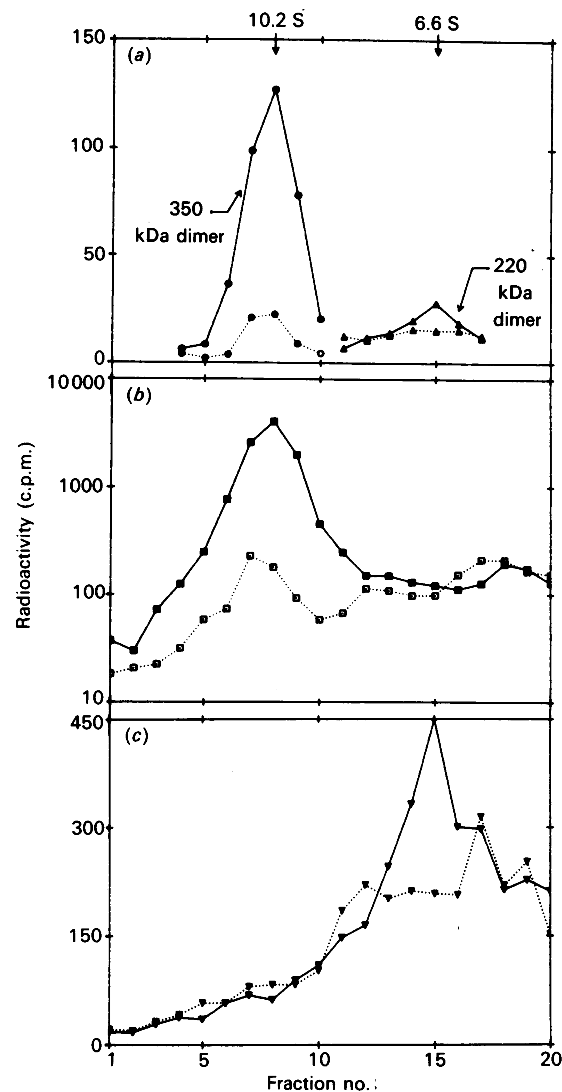

Fig. 7. Effect of membrane reduction on the sedimentation characteristics of rat adipocyte plasma-membrane insulin-dependent kinase activity

Rat adipocyte plasma membrane was incubated with or without 10 mM-DTT for 30 min at room temperature. Membranes were washed, solubilized, and velocity-sedimented; gradient fractions were phosphorylated, electrophoresed and analysed for incorporated phosphate as in Fig. 3. Panel (*a*) shows receptor autophosphorylation with or without insulin in gradient fractions around 10.2 S (350 kDa dimer) in the absence of reduction and 6.6 S (220 kDa monomer) after membrane reduction. Panels (*b*) and (*c*) show histone 2B kinase activity across the gradient with unreduced and reduced membranes respectively. Panel (*a*) shows counts incorporated by autophosphorylation into receptor bands identified autoradiographically after SDS/PAGE. Bands corresponding to the M_r of the receptor dimer were seen only in the absence of DTT and exhibited an apparent sedimentation coefficient of 10.2 S. Likewise, those bands identified at the M_r of the monomer were seen only when membrane was treated with 10 mM-DTT and only at the position of 6.6 S within the gradient. Shown are counts incorporated into dimer (○, ●) and monomer (△, ▲) bands in the absence and presence of 100 nM-insulin respectively. Panel (*b*) shows the ability of gradient fractions from unreduced membrane to phosphorylate histone 2B in the absence and presence of 100 nM-insulin (□, ■). Panel (*c*) shows the histone kinase activity of gradient fractions of velocity-sedimented solubilized membrane, previously reduced by 10 mM-DTT, in the absence and presence of 100 nM-insulin (▽, ▼).

Table 3. Effect of reduction on the sedimentation properties of rat adipocyte receptor kinase activity

As described in Fig. 7, rat adipocyte plasma membranes were incubated for 30 min at room temperature with or without 10 mM-DTT. After removal of reductant, membranes were solubilized and velocity-sedimented, and the gradient fractions were phosphorylated and electrophoresed in accordance with standard procedures. Identification of phosphorylated receptor and histone bands and quantification of incorporated radioactive phosphate were accomplished as in Table 2. Also shown is the ratio of insulin-dependent phosphate incorporation into histone relative to that into receptor β-subunit, for a representative result of triplicate experiments.

DTT		Radioactivity (c.p.m./peak)			
		−Insulin	+Insulin	Specific	Ratio
0	Receptor dimer				
	Receptor	67	376	309	
	Histone	725	10201	9476	30.7
10 mM	Receptor monomer				
	Receptor	59	80	21	
	Histone	826	1328	502	23.5

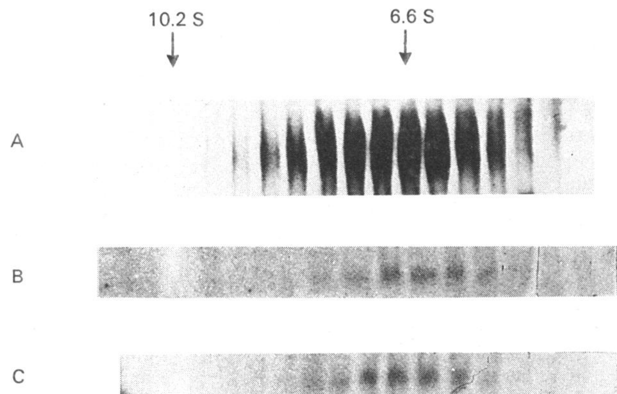

Fig. 8. Effect of phosphorylation, insulin and Mg^{2+} on the sedimentation coefficient of insulin-receptor-monomer kinase activity

Rat adipocyte plasma membranes were incubated with 10 mM-DTT for 30 min before solubilization of receptors and sucrose-gradient velocity sedimentation and fractionation of the gradients. Fractions surrounding the 6.6 S peak were pooled. Panel A shows the result of phosphorylating the monomer pool before repeated sucrose-gradient velocity sedimentation. Fractions spanning the expected position of receptor monomers and dimers were submitted to reducing SDS/PAGE. Panels B and C show the result of incubating pooled monomer with 10 mM-$MgCl_2$, 2 mM-$MnCl_2$, with or without 150 nM-insulin respectively. Samples were then velocity-sedimented on gradients containing an equal amount of metal or metal and hormone respectively. Fractions spanning the region 6.6–10.2 S were then phosphorylated by the standard protocol and evaluated by reducing SDS/PAGE. Indicated sedimentation values are estimated from marker enzyme peak positions. All three panels show the portion of the gels corresponding to the M_r of reduced β subunit, which represented the only band of insulin-stimulated phosphorylation.

8 indicate that any non-covalent dimerization associated with either phosphorylation of the receptor monomer or the binding of insulin to the receptor monomer is not stable enough to alter the sedimentation characteristics of the receptor. In the second place, there is no effect of dilution of the receptor monomer on the kinase activity of the monomer peak, as is shown in Tables 2 and 4; this result argues against the existence of non-covalently associated monomers.

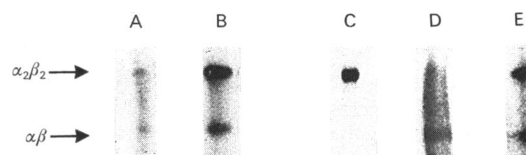

Fig. 9. Effect of dilution on phosphorylation of partially reduced solubilized receptors

Rat adipocyte plasma-membrane receptors isolated by elution from WGA were incubated with 1 mM-DTT for 20 min and subsequently incubated with or without 100 nM-insulin for 20 min. Phosphorylation was initiated as described in the Materials and methods section and effected for 3 min at which time it was terminated by addition of *N*-ethylmaleimide and sample buffer. Lanes A and B show control and insulin-stimulated phosphorylation respectively. Lanes C, D and E represent 3 min phosphorylations of rat adipocyte insulin-receptor dimers and monomers (prepared as in Fig. 3 for turkey erythrocyte membranes) and partially reduced (1 mM-DTT) WGA-purified rat adipocyte insulin receptor (prepared as in lanes A and B) respectively. Samples were bound to immobilized insulin and phosphorylated as described in the Materials and methods section.

Table 4. Effect of dilution on phosphorylation of partially reduced solubilized receptor

Samples (25 ml) of WGA-purified receptor partially reduced in 0.1% Triton X-100 solution as in lanes A and B of Fig. 9 were or were not diluted to 10 times their original volume, incubated with 100 nM-insulin and phosphorylated as before for 1 or 3 min as indicated. Phosphorylated receptor bands were excised and counted for radioactivity. Shown in the first four columns are the total radioactivity (c.p.m., S.D. ±1%) and the ratio of counts incorporated into monomers and dimers as a function of time and dilution. Column 5 shows those counts incorporated into receptor bands when partially reduced receptor was pre-bound to insulin–agarose and then phosphorylated on the immobilizing support for 3 min. Shown is a representative set of results from three experiments.

	Radioactivity (c.p.m./band)				
	1 min	1 min, dilute	3 min	3 min, dilute	3 min, insulin–agarose
Monomer	44	129	143	242	28
Dimer	126	302	338	572	61
Ratio	0.347	0.426	0.425	0.425	0.475

The evidence that the monomers, at the concentrations used in these experiments, do not form dimers that are detectable by sucrose-density-gradient centrifugation and are not affected by dilution in their kinase activity is a strong argument that the monomers themselves possess protein kinase activity.

The result that covalent association of monomers by Class I disulphides is not necessary for insulin stimulation of the kinase activity has been described previously (Shia *et al.*, 1983; Fujita-Yamaguchi & Kathuria, 1985; Pike *et al.*, 1986). The present work shows, however, that the $\alpha\beta$ monomer by itself has the capacity to bind insulin on the α chain and transduce the effect to the kinase on the β chain, presumably through the single trans-membrane strand (Ullrich *et al.*, 1985).

These results are in agreement with those obtained by the Rosen group (Herrera *et al.*, 1988; Villalba *et al.*, 1989) with the cytoplasmic domain of the human insulin-receptor β subunit; the latter protein is a monomer, and its autophosphorylation is independent of protein concentration.

There remains the question of the lack of tyrosine kinase activity of the monomers present in turkey erythrocyte membranes before reduction. Obviously, these species are different from the monomers produced by reduction of dimers, because they lack kinase activity. There are several interpretations of this finding. One is that native monomers do lack kinase activity; the monomers described here with kinase activity would represent partially denatured (manipulated) species whose properties are unrelated to those of native receptor monomers. Another possibility is that the naturally occurring $\alpha\beta$ monomers have been altered either by proteolysis (Roth *et al.*, 1983; Massague *et al.*, 1981) or by disulphide-bond rearrangement (Shia *et al.*, 1983) and have lost kinase activity; the monomers with kinase activity described in the present work would then represent the true state of native receptor monomers. One cannot distinguish between these possibilities.

In any event, the $\alpha\beta$ monomer, obtained by reduction of dimers in the membrane, is active in insulin binding and insulin-dependent protein kinase activity.

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