Hormone-triggered conformational changes within the insulin-receptor ectodomain: requirement for transmembrane anchors

Ralf-Rüdiger FLÖRKE^{*1}, Kerstin SCHNAITH^{*}, Waltraud PASSLACK^{*}, Marc WICHERT^{*}, Lothar KUEHN^{*}, Marlies FABRY[†], Matthias FEDERWISCH[‡] and Hans REINAUER^{*}

^{*}Deutsches Diabetes-Forschungsinstitut an der Heinrich Heine-Universität, Klinische Biochemie², Auf'm Hennekamp 65, 40225 Düsseldorf, Germany, [†]Deutsches Wollforschungsinstitut, Veltmanplatz 8, 52062 Aachen, Germany, and [‡]Institut für Biochemie der Rheinisch-Westfälischen Technischen Hochschule, Klinikum, Pauwelsstr. 30, 52057 Aachen, Germany

Interaction between two $\alpha\beta$ half-receptors within the $(\alpha\beta)_2$ holoreceptor complex is required for insulin binding with high affinity and for insulin-triggered changes of size and shape. To understand the underlying structure–function relationship, two truncated receptor constructs have been characterized. Reduction in the Stokes radius and increase in the sedimentation coefficient, which are characteristic for wild-type receptors, were entirely lacking for the recombinant human insulin receptor (HIR) ectodomain (HIR-ED). Stokes radii of about 5.8 nm and sedimentation coefficients of 10.2 S were found for both insulin-bound and free HIR-EDs. However, attaching the membrane anchors to the ectodomain, as with the recombinant membrane-anchored ectodomain (HIR-MAED) construct, was sufficient to restore not only high-affinity hormone binding but also the marked insulin-inducible alterations in hydrodynamic properties. The Stokes radii of HIR-MAED complexes, as assessed by non-denaturing PAGE, decreased upon insulin binding from

9.5 nm to 7.9 nm. In parallel, the sedimentation coefficient was increased from 9.0 S to 9.8 S. CD and fluorescence spectroscopy of HIR-MAED revealed only minor insulin-induced changes in the secondary structure. Similarity with wild-type receptors has also been demonstrated by the differential insertion of insulin-bound and free HIR-MAED complexes into artificial bilayer membranes of Triton X-114. The results are consistent with a model of receptor function that ensures a global insulin-triggered reorientation of subdomains within the ectodomain moieties while the secondary structure is essentially retained. For the rearrangement of such subdomains, the transmembrane anchors confer essential structural constraints on the receptor ectodomain.

Key words: circular dichroism, fluorescence spectroscopy, hydrodynamic properties, non-denaturing electrophoresis, Triton X-114.

INTRODUCTION

The insulin receptor gene encodes for a membrane-inserted polypeptide that is further processed subsequent to translation. This polypeptide is cleaved into the α - and β -subunits, which remain coupled by disulphide bonds. Formation of disulphide bonds between two α -subunits leads to the heterotetrameric $(\alpha\beta)_2$ holoreceptor complex. The hormone-binding α -subunits are entirely extracellular whereas the β -subunits span the plasma membrane each by a single helix of hydrophobic amino acids. X-ray studies have elucidated the structure of the major cytoplasmic portion of the β -subunit as the typical architecture of tyrosine kinases (reviewed in [1–8]).

Most members of the membrane-receptor tyrosine kinase family accomplish activation by dimerization of two half-receptors upon ligand binding (reviewed in [9]). Non-covalent association is crucial for the arrangement of the respective cytoplasmic tyrosine kinase domains in a functional orientation. However, the insulin receptor, as well as the closely related insulin-like growth factor 1 receptor, and the insulin-related receptor consist of two permanently coupled $\alpha\beta$ half-receptors and thus require a different mechanism for signal transduction across the plasma membrane. There is convincing evidence that autophosphorylation of the β -subunits is a crucial step for kinase activation towards insulin receptor substrate proteins (recently

reviewed in [5,10,11]). It has become an accepted concept that one function of the extracellular receptor portions is to keep the catalytic loop of one tyrosine kinase subunit unavailable for the partner kinase domain. According to this model, insulin binding, which has been elucidated as a co-operative process [4], stabilizes a receptor conformer in which such constraints are removed. This may be achieved by the coupling of one insulin molecule to both α -subunits of the heterotetrameric receptor complex [12]. Hormone-binding sites have been attributed to the L1–Cys-rich–L2 domain (amino acids 1–468; see [13]) and a relatively short segment further downstream (amino acids 704–719) [14–20].

Indirect evidence for distinct conformations of insulin-bound and free $(\alpha\beta)_2$ receptor complexes has been provided by several laboratories. More directly, biophysical studies have revealed a markedly different size and shape for each receptor form [21,22]. In contrast to $(\alpha\beta)_2$ receptors, $\alpha\beta$ half-receptors, whose affinity for insulin is reduced by one or two orders of magnitude and whose tyrosine kinase is not stimulated by insulin binding [22–24], do not exhibit such changes [22]. This indicates that the conformational changes which underlie the alterations in size and shape may be essential for normal receptor function [22]. The present study aimed to localize the receptor domains involved. Knowledge of such structure and function relationships should shed additional light on the various steps in receptor function, i.e.

Abbreviations used: DSS, disuccinimidyl suberate; HIR, human insulin receptor; ED, ectodomain; WT, wild-type; MAED, membrane-anchored ectodomain.

¹ To whom correspondence should be addressed (e-mail ralf.floerke@ddfi.uni-duesseldorf.de).

² This department is now named Abteilung für Klinische Biochemie und Pathobiochemie.

high-affinity insulin binding, signal transmission from the extracellular receptor moiety to the cytoplasmic tyrosine kinase domain and tyrosine kinase activation. Here it is demonstrated that structural changes proximal to the cytoplasmic domain cause marked alterations in the sizes and shapes of ($\alpha\beta$)₂ insulin receptors. Within this scenario, the transmembrane anchors play a crucial role.

EXPERIMENTAL

Construction of truncated insulin-receptor genes

The wild-type human insulin receptor (HIR-WT) gene (minus exon 11) was excised from the plasmid vector CVN-HIR (kindly donated by A. Ullrich, Max Planck-Institut für Biochemie, Martinsried, Germany) using restriction enzymes *Hind*III (5' of the insulin-receptor gene) and *Drd*I. Before ligation with T4 ligase (Roche) into the *Hind*III and *Eco*RV sites of the vector pcDNA3 (Invitrogen), the *Drd*I site of the receptor gene was made blunt-ended by digestion of the 3'-overhangs with T4 DNA polymerase (Clontech/ITC) according to standard procedures [25]. The resulting vector, pcDNA3-HIR-WT (9835 bp), was used for transient expression of wild-type receptors in HEK-293 cells and for the construction of the truncated receptor forms.

For the construction of the recombinant HIR ectodomain (HIR-ED) gene and the gene encoding the HIR membrane-anchored ectodomain (HIR-MAED), stop codons were introduced after the DNA triplets coding for Lys-917 and Gly-947, respectively. This was achieved by priming PCR products with the plus-strand oligonucleotide 5'-CAT CTC TGC GTC TCC CGC AAG CAC-3' and the minus-strand oligonucleotides 5'-CCA TAT GGG CCC TTA TTT TGC AAT ATT TGA CGG GA-3' (for construction of the HIR-ED gene) or 5'-CCA TAA GGG CCC TTA CCC ATC TGG CTG CCT CTT TCT-3' (for construction of the HIR-MAED gene; the stop-codon is in bold, the restriction sites for *Esp*3I and *Bsp*120I, respectively, are given in italics; additional bases of the 5'-ends were added to facilitate restriction). The vector pcDNA3-HIR-WT (0.1 ng) was used as a template for PCR; 35 cycles were performed with an annealing temperature of 66 °C. The PCR products were digested with *Esp*3I and *Bsp*120I and purified by agarose-gel electrophoresis. The DNA was extracted from the gel with the QIAquick gel extraction kit (Qiagen) and subsequently ligated into the corresponding restriction sites of the vector pcDNA3-HIR-WT. Sequence analysis of the entire inserts confirmed the desired nucleotide sequences within the newly synthesized vectors pcDNA3-HIR-ED (8258 bp) and pcDNA3-HIR-MAED (8348 bp).

A similar receptor construct embraced the first 909 amino acid residues from the receptor ectodomain and two residues in addition, Ala and Arg, which had been added at the C-terminus as a result of the cloning procedure [26–28]. We also engineered this receptor form. Non-denaturing PAGE did not reveal any principal differences between this receptor type and HIR-ED (results not shown).

Expression of insulin receptors

About 2×10^6 HEK-293 cells were seeded on to a 10 cm plate and incubated overnight in 10 ml of growth medium (Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, penicillin and streptomycin). DNA (20 µg) was mixed with 0.2 M CaCl₂ in 0.5 ml. The formation of calcium phosphate crystals was initiated by the addition of 0.5 ml of 50 mM Bes/280 mM NaCl/1.5 mM Na₂HPO₄, pH 6.95. After 20 min at

room temperature, the suspension was added dropwise to the cell medium. Further incubation was at 35 °C in 3 % CO₂. After 18 h, the plates were washed twice with 5 ml of PBS and subsequently supplemented with 10 ml of transfection medium (one-third growth medium/two-thirds growth medium containing high-glucose Dulbecco's modified Eagle's medium). Further incubation was at 37 °C in 5 % CO₂.

Insulin-receptor purification

The 100 000 g supernatant of the solubilized cells (50 mM Hepes, pH 7.5/150 mM NaCl/10 % glycerol/1.5 mM MgCl₂/1 mM EDTA/100 mM NaF/1 mM PMSF/10 µg/ml aprotinin) or the cell supernatant was subjected to insulin affinity chromatography [22]. Receptors eluted with 50 mM sodium acetate, pH 5.0/1 M NaCl/0.1 % Triton X-100 were concentrated using wheatgerm agglutinin-affinity chromatography. Receptor protein, eluted with 300 mM *N*-acetylglucosamine was concentrated with Centricon 100 microconcentrators (Millipore/Amicon). The samples were used for sucrose-density centrifugation (see below).

Competition binding studies of purified receptors were performed in solution as described in [22]. Half-maximal inhibitory constants (IC₅₀) were calculated using GraphPad Prism version 3.02 (GraphPad Software).

PAGE

SDS/PAGE was performed with a 4–30 % acrylamide gradient slab gel (acrylamide/bisacrylamide, 37.5) containing 0.75 M Tris/HCl, pH 8.8. Broad-range protein markers were from New England Biolabs. Relative amounts of silver-stained proteins were assessed on a Kodak Image Station 440 using Kodak Digital Science 1D (3.01) software.

Non-denaturing gradient gels [22] were poured from 3 % acrylamide (acrylamide/bisacrylamide, 100) and 43 % acrylamide (acrylamide/bisacrylamide, 37.5) in 100 mM Tris/HCl, pH 8.8, containing 0.1 % Triton X-100. Running buffer was 6.25 mM Tris/HCl, 50 mM glycine and 0.1 % Triton X-100. For estimating Stokes radii, reference proteins from the electrophoresis calibration kit (Amersham Pharmacia Biotech) were used. Protein bands were visualized by silver staining. For the analysis of ¹²⁵I-labelled insulin-coupled receptor complexes, slab gels were dried and exposed to X-Omat AR film (Kodak).

Cross-linking of receptor–insulin complexes

For cross-linking experiments, human Tyr^{A14}-¹²⁵I-labelled insulin was separated from albumin by microfiltration with Microcon 30 microconcentrators (Millipore/Amicon). Cross-linking of receptor–insulin complexes was performed with 125 µM disuccinimidyl suberate (DSS; Pierce) [29]. The reaction was quenched by the addition of 0.5 M Tris/HCl, pH 7.4. The samples were washed twice (in 10 mM Tris/HCl, pH 7.4/0.025 % Triton X-100) with Microcon 100 concentrators and subjected to analysis.

Gel filtration

Superdex 200 matrix was used for gel filtration in conjunction with the SMART chromatography system (Amersham Pharmacia Biotech). Aliquots of 40 pmol of receptor protein were bound to non-saturating amounts of ¹²⁵I-labelled insulin (1 pmol) and to saturating amounts of unlabelled insulin (0.5 nmol). Cross-linking with DSS was performed as described above. Both samples were mixed and concentrated by microfiltration with Microcon 100. Samples were subjected to chromatography in a

volume of 5 μ l. Fractions of 20 μ l were eluted with 50 mM Tris/HCl/150 mM NaCl/0.1 % Triton X-100, pH 7.4, with a flow rate of 50 μ l/min and analysed by non-denaturing PAGE. Elution profiles of hormone-bound and free receptor complexes were constructed after densitometric scanning of the silver-stained gels. Autoradiography was used to verify congruence and concentration profiling of 125 I-labelled insulin-bound receptor complexes.

The distribution coefficient, K_{av} , was calculated from the function $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 is the void volume and V_t is the total volume. Linearity of the plot $(-\log K_{av})^{1/2}$ against Stokes radius was found for the reference proteins thyroglobulin (8.6 nm), γ -globulin (5.5 nm) and ovalbumin (2.7 nm; all from Amersham Pharmacia Biotech).

Velocity sedimentation

Hormone-cross-linked receptor complexes (HIR-WT with HIR-ED, or HIR-MAED with HIR-ED) were loaded on to a 4 ml linear (5–20 %) sucrose density gradient (in 50 mM Tris/HCl, pH 7.4/150 mM NaCl/0.1 % Triton X-100) in a volume of 50 μ l. In addition, each sample contained catalase as an internal standard. Ultracentrifugation was performed with a Beckman SW 60 rotor for 11 h at 300 000 *g*. Sedimentation coefficients were measured with reference to standard proteins, BSA ($s_{20,w} = 4.3$ S), γ -globulin ($s_{20,w} = 7.1$ S) and catalase ($s_{20,w} = 11.3$ S).

Triton X-114 phase partition

HIR-MAED (20 pmol) was incubated in 25 μ l of 50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 % Triton X-114 (Roche) in the presence or absence of 0.2 nmol of insulin. After 16 h at 5 °C the samples were kept at 37 °C for 1 min and centrifuged at 10 000 *g* for 1 min. The phases were separated and reconstituted to 25 μ l. Equal volumes of each fraction were analysed by SDS/PAGE and silver staining.

Protein analysis

HIR-MAED protein was quantified by protein micro mass analysis (kindly carried out by H. E. Meyer, Institut für Physiologische Chemie der Ruhruniversität Bochum, Bochum, Germany). The amounts of HIR-WT and HIR-ED were assessed by co-electrophoresis with HIR-MAED samples of known receptor concentration and by subsequent silver staining.

Insulin concentrations were determined spectrophotometrically (molar extinction coefficient, $\epsilon_{276} = 6187 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

CD

CD measurements were carried out in 10 mM Tris/HCl/0.025 % Triton X-100, pH 7.4, on an AVIV (Lakewood, NJ, U.S.A.) 62DS spectrometer, calibrated according to [30]. The spectral bandwidth was 2 nm, and the temperature 27 °C.

Spectra of (1) insulin alone and (2) the mixture of receptor and insulin were recorded. To calculate the molar (residue) ellipticity of the insulin-bound receptor (IR), the spectrum of insulin alone (I) was subtracted from the spectrum of the receptor/insulin mixture (IRI):

$$[\Theta]_{\text{IR}}^{\text{MRW}} = \left(\theta_{\text{IRI}} - \theta_1 \frac{c_{12}l_2}{c_{11}l_1} \right) \frac{100}{c_{12}l_2} \quad (1)$$

where l is the pathlength, θ is the measured ellipticity, MRW is mean residue weight, and c , the molar concentration of peptide bonds, is calculated as follows:

$$c = c[g/l]/\text{MRW} \quad (2)$$

The molar (residue) ellipticity of the receptor/insulin mixture (IRI) is calculated as follows:

$$[\Theta]_{\text{IR}}^{\text{MRW}} = \theta_{\text{IRI}} \frac{100}{c_{\text{IRI}2}l_2} \quad (3)$$

In this case c is calculated as follows:

$$c_{\text{IRI}2} = \frac{c_{\text{tot}}[g/l]}{\text{MRW}_I \frac{c_I[g/l]}{c_{\text{tot}}[g/l]} + \text{MRW}_R \frac{c_R[g/l]}{c_{\text{tot}}[g/l]}} \quad (4)$$

where

$$c_{\text{tot}}[g/l] = c_I[g/l] + c_R[g/l] \quad (5)$$

Fluorescence spectroscopy

Steady-state fluorescence was measured on a Spex Fluorolog 211 photon-counting spectrometer (Spex Industries, Edison, NJ, U.S.A.). The excitation wavelength was 295 nm and the bandwidth was 1.8 nm. The system provides corrections for changes in the lamp intensity and for the spectral sensitivity of the emission monochromator and photomultiplier.

For time-resolved fluorescence, intensity and anisotropy decay were measured in single-photon-counting mode with a model 199 spectrometer (Edinburgh Instruments, Edinburgh, U.K.).

To avoid interference by tyrosine fluorescence, a comparably long excitation wavelength (300 nm) was chosen (bandwidth, 12 nm). Moreover, at this excitation wavelength, there is no fluorescence emission by insulin due to the absence of tryptophan residues in this protein.

Data handling and the iterative non-linear least-squares fit of the decays were accomplished by a program supplied by Edinburgh Instruments. Correlation times of insulin-bound HIR-MAED complexes were found to be greater than 10 times the mean fluorescence lifetime, $\langle \tau \rangle$, and therefore had to be considered as approximations [31]. A more detailed discussion of the methodology was presented earlier [32].

RESULTS

SDS/PAGE and hormone binding of wild-type and truncated insulin receptors

Wild-type receptors (HIR-WT), as well as truncated receptor forms embracing either all the amino acid residues of the soluble ectodomain (HIR-ED) or an ectodomain receptor moiety elongated by the transmembrane anchors (HIR-MAED), were expressed transiently in HEK-293 cells. Each receptor species was purified by insulin affinity chromatography and sucrose density-gradient centrifugation. Molecular masses of 420, 280 and 270 kDa were determined by SDS/PAGE for isolated HIR-WT, HIR-MAED and HIR-ED, respectively (Figure 1). The apparent molecular masses correspond to the heterotetrameric subunit stoichiometry [26–28,33,34]. α -Subunits from all receptor subtypes were of comparable size (results not shown). IC_{50} values of about 9 pM were determined for HIR-MAED and HIR-WT, whereas HIR-ED complexes exhibited a substantially lower affinity, as demonstrated by the relatively high IC_{50} value of 2.2 nM (results not shown explicitly).

Sizes and shapes of hormone-bound and free receptor complexes

Upon non-denaturing PAGE, silver staining revealed single-banding proteins for HIR-WT, HIR-MAED and HIR-ED

Figure 1 Non-reducing SDS/PAGE of purified insulin receptors

Purified wild-type insulin receptors (WT, 0.3 pmol), recombinant MAED complexes (1 pmol) and recombinant ED complexes (1 pmol) were subjected to SDS/PAGE and visualized by silver staining. The identity of the silver-stainable proteins had been confirmed by demonstrating specific hormone binding after cross-linking to ¹²⁵I-labelled insulin, separation by SDS/PAGE and autoradiography (results not shown). The relative migration distances of myosin (212 kDa) and β -galactosidase (116 kDa) are indicated.

preparations (Figure 2, lanes a). Although HIR-MAED receptor complexes (1886 residues) lack almost the entire cytoplasmic receptor moiety, they co-migrated with the wild-type receptor species (2678 residues). A Stokes radius of 9.5 nm was determined for the membrane-anchored receptor forms. The unexpected size of HIR-MAED does not reflect retarded migration caused by a loss of acidic amino acids as the pI value of the truncated receptor form (6.0) is only slightly higher than that calculated for wild-type receptors (5.6; calculated with PHYSCHEM from the PC/Gene package). Moreover, gel filtration also revealed similar Stokes radii for HIR-WT and HIR-MAED (see below). Only the additional loss of the transmembrane anchors led to a substantial decrease in Stokes radius (5.8 nm), as shown for the HIR-ED receptor form (1826 residues).

Similar to ($\alpha\beta$)₂ receptor complexes from human placenta [22], marked insulin-inducible alterations in size and shape were also found with recombinant human ($\alpha\beta$)₂ wild-type receptor com-

Figure 3 Gel filtration of hormone-bound and free insulin receptors

Samples of insulin-free and cross-linked insulin-bound HIR-MAED or HIR-ED complexes were prepared as described in the Experimental section, and were subjected to chromatography on Superdex 200. Fractions were analysed by non-denaturing PAGE. Protein-concentration profiles were calculated from densitometric scanning of silver-stained gels and autoradiograms. Concentration was measured with reference to that in the fraction with the highest receptor concentration. The peak elution fractions of the reference proteins thyroglobulin (8.6 nm) and γ -globulin (IgG, 5.5 nm) are indicated. Elution profiles of HIR-WT were congruent with those of HIR-MAED and are therefore not shown.

plexes purified from HEK-293 cells (Figure 2). Binding of non-saturating amounts of ¹²⁵I-labelled insulin and subsequent cross-linking allowed us to detect a labelled complex of 7.9 nm by autoradiography (Figure 2, lanes c). Detection of a corresponding band by silver staining (Figure 2, lanes d) required receptor preincubation in the presence of a saturating insulin concentration (10 μ M). Apparently, treatment with the bifunctional cross-linker DSS alone was without effect (Figure 2, lanes b). As shown in Figure 2, migration of HIR-ED was not accelerated

Figure 2 Non-denaturing PAGE of hormone-bound and free insulin-receptor complexes

Purified insulin-receptor protein (2 pmol) representing wild-type receptors (WT), the recombinant ED and the MAED were incubated with 10 fmol of ¹²⁵I-labelled insulin (lanes c), or 4 pmol of protein were incubated with 10 fmol of ¹²⁵I-labelled insulin to which 1 nmol of non-labelled insulin had been added for competition (lanes d). Receptor samples were treated with or without DSS. For visualization, separated proteins were stained with silver. Radioactively labelled protein complexes were identified by autoradiography. Control experiments demonstrated that derivatization with DSS did not affect mobility of the receptor complexes (lanes a versus b). The relative migration distances of thyroglobulin and ferritin are indicated.

Figure 4 Velocity centrifugation of hormone-bound and free insulin receptors

Insulin-free receptors and receptor complexes cross-linked to insulin were prepared as described in the Experimental section. Samples subjected to sucrose density-gradient centrifugation were mixtures of catalase as an internal reference with HIR-MAED and HIR-ED, or with HIR-WT and HIR-ED. After centrifugation, fractions of 100 μ l (starting with the top fraction) were analysed electrophoretically by non-denaturing PAGE. For analysis of the distribution profile of insulin-free ($\diamond$) and insulin-bound ($\blacklozenge$) HIR-MAED complexes, and insulin-free ($\square$) and insulin-bound ($\blacksquare$) HIR-ED complexes, the silver-stained gel and the autoradiographs were scanned on a Kodak Image Station. The intensity of each band was measured with reference to the fraction with the highest receptor concentration. The elution profiles of hormone-free ($\triangle$) and hormone-bound ($\blacktriangle$) HIR-WT are from a separate experiment.

when complexed with insulin. Rather, the slight bandshift after binding of insulin may indicate a small increase in the Stokes radius. In conclusion, one of the domains located further downstream of the ectodomain is obligatory for the insulin-triggered changes that are characteristic of wild-type receptor complexes.

To localize receptor domains that are required for the insulin-inducible alterations in size and shape, we examined recombinant receptor complexes that also possessed the transmembrane domain HIR-MAED. The addition of transmembrane anchors was found to restore the insulin-inducible changes in size and shape completely, as demonstrated by non-denaturing PAGE (Figure 2).

In addition to non-denaturing PAGE, the influence of insulin binding on size and shape of the receptor species was examined by gel filtration (Figure 3). A Stokes radius of 6.0 nm was determined for both insulin-bound and free HIR-ED complexes. In accordance with the results obtained by non-denaturing PAGE, Stokes radii of 7.8 nm were determined for complexes of HIR-WT and HIR-MAED with insulin. Insulin-free complexes of HIR-WT and HIR-MAED, however, may have reached the exclusion limit of the gel-filtration matrix. This may explain the relatively low Stokes radius (8.1 nm) determined for these receptor species by gel filtration. Interestingly, Stokes radii of insulin-receptor complexes have been reported to vary as a function of the matrix used for gel-filtration chromatography [22].

Velocity sedimentation of insulin-bound versus free receptor complexes

Velocity-sedimentation centrifugation demonstrated that the sedimentation coefficient of HIR-ED was 10.2 S in both the hormone-bound and free states (Figure 4). In contrast, sedimentation coefficients of the membrane-anchor-attached receptor forms were altered markedly by insulin binding. For HIR-WT receptor complexes, the increase in S value from 10.2 S (insulin-free form) to 11.2 S (insulin-bound state) corresponded with the change determined previously for $(\alpha\beta)_2$ full-length receptors from human placenta [22]. HIR-MAED receptor species displayed a sedimentation coefficient of 9.0 S, which was shifted to 9.8 S upon insulin binding.

Differential interaction of hormone-bound and free receptor proteins with Triton X-114

Detergent bilayer membranes, which co-segregate above the cloud-point of Triton X-114 [35], have been found to incorporate insulin-free $(\alpha\beta)_2$ receptor complexes [36]. In contrast, the majority of insulin-bound species preferred the detergent-depleted phase.

To localize the receptor domains that may cause the differential partitioning, HIR-MAED complexes were examined. Similar to wild-type receptors, more than 90 % of insulin-free HIR-MAED complexes were detected in the detergent-bilayer phase. This was

Figure 5 Differential insertion of insulin receptors into Triton X-114 membranes

Samples of purified HIR-ED and HIR-MAED were made 1% in Triton X-114 and incubated in the absence or presence of saturating amounts of insulin. Triton X-114 phase partition was initiated by raising the temperature to 37 °C. After centrifugation, the two phases were separated and the volumes were adjusted. Identical aliquots were applied to SDS/PAGE under disulphide-reducing conditions. After silver staining, intensities of the α -subunit bands were measured by densitometric scanning. The distributions of insulin receptors between the detergent-depleted phase (white bars) and the membrane phase (hatched bars) are given as percentages.

nearly reversed upon insulin binding, as shown in a typical experiment (Figure 5). Less than 20% of insulin-bound HIR-MAED complexes were found to prefer the detergent-rich phase. As may be expected, HIR-ED complexes, whether in the insulin-bound or free state, preferred the detergent-depleted phase and thus behaved as typical water-soluble proteins.

The data indicate that, after insulin binding, a change in physico-chemical properties proximal to the cytoplasmic receptor moiety interferes with detergent-membrane embedding. Steric hindrance by ectodomain portions after conformational reorganization is a likely explanation.

CD spectroscopy of HIR-MAED

CD spectroscopy with HIR-MAED is restricted to wavelengths > 205 nm by the presence of detergent micelles. Due to the small amount of receptor protein available, only far-UV analysis was carried out.

As shown in a comparative representation of the spectra of the recombinant ectodomain [32] and HIR-MAED, the shapes are markedly similar (Figure 6). This indicates similar secondary structures of both receptor forms.

To assess whether the secondary structure was altered by insulin binding, hormone-bound and free HIR-MAED were examined. The spectra of HIR-MAED were recorded in the absence (spectrum 2) or presence (spectrum 3) of insulin (Figure 7). The spectrum of insulin-bound HIR-MAED (Figure 7, spectrum 1) was calculated by subtracting the spectrum of free insulin (results not shown) from spectrum 3. The spectra are not splined in order to show the signal-to-noise ratio.

To assess which of the two partners contribute mainly to the observed changes, the undisturbed HIR-MAED spectrum was also subtracted from spectrum 3 (results not shown). The resulting spectrum was incompatible with the known structural stability of the hormone ([32] and M. Federwisch and A. Wollmer, unpublished work). In conclusion, the alterations in the CD

Figure 6 Far-UV CD spectra of insulin receptors

The spectrum of the recombinant ectodomain (spectrum 1; $c = 3.5 \mu\text{M}$; data obtained from [32]) was compared with that of HIR-MAED (spectrum 2; $c = 2.8 \mu\text{M}$).

spectra were due mainly to structural changes within the receptor construct, whereas possible structural changes within the peptide hormone had only a minor influence.

Upon insulin binding there was a small change in the CD spectrum (Figure 7), which largely corresponds to that seen with the recombinant ectodomain [32].

Fluorescence spectroscopy of HIR-MAED

The steady-state fluorescence spectrum of hormone-free HIR-MAED peaked at 335 nm with a full width at half-maximum (FWHM) of 58 nm (Figure 8). After insulin binding, a minor decrease in fluorescence intensity, which was due partially to the dilution by insulin, was accompanied by a small blue shift. Such alterations indicate a decrease in the polarity in the environment of some tryptophan residues. This is also reflected by a decrease in the mean fluorescence lifetime from $\langle \tau \rangle = 4.10 \text{ ns}$ to 3.76 ns (Table 1). Similarly small alterations have been found for the purified recombinant ectodomain [32]. The data further support the hypothesis that prominent tryptophan-affecting structural changes occur only in the cytoplasmic receptor moiety [37].

Rotational diffusion during the lifetime of the excited state displaces the emission dipole of the fluorophore, which results in a decay of the anisotropy. The rotational correlation time, ϕ , is a measure of its velocity (the slower the decay the higher the value of ϕ). For the insulin-free HIR-MAED complex a ϕ value of 9.9 ns was determined. A second correlation could not be fitted to the decay. Inspection of its time course showed an initial

Figure 7 The effect of insulin on the CD spectrum of HIR-MAED in the far-UV

Spectra of HIR-MAED (2.6 μ M) were recorded in the absence (spectrum 2) or presence (spectrum 3) of insulin (45.8 μ M). Spectrum 1 was plotted after subtraction of the separately recorded insulin spectrum (not shown) from spectrum 3.

Figure 8 Steady-state fluorescence spectra

Spectra of HIR-MAED were recorded in the absence (spectrum 1) or presence (spectrum 2) of insulin as described in the Experimental section. Concentrations are given in the legend of Figure 7.

Table 1 Fluorescence lifetime and anisotropy decay of insulin-bound or free HIR-MAED complexes

Protein concentrations are given in the legends of Figures 6 and 7. Data were recorded at an excitation wavelength of 300 nm with a bandwidth of 12 nm. Fluorescence emission was passed through DUG 11 and WG 320 filters. The lamp pulse was recorded at 340 nm using a diluted suspension of Ludox (Du Pont, Wilmington, DE, U.S.A.). All measurements were carried out at room temperature. Values for $\langle \tau \rangle$ and ϕ are given in ns, B is expressed as a percentage. Total fluorescence intensity decays were fitted to the equation $s(t) = b_0 + \sum S b_i \exp(-t/\langle \tau \rangle_i)$ with $i = 1, 2, \dots, n$. b_i and τ_i are the amplitude and lifetime of the i th excited state, respectively, and χ^2 is the reduced Chi-squared. B_i and the mean fluorescence lifetime, $\langle \tau \rangle$, were calculated according to $\langle \tau \rangle = \sum B_i \tau_i = \sum [b_i \tau_i / \sum b_i \tau_i]$. Anisotropy decays were fitted to the equation $r(t) = \sum r_i \exp(-t/\theta_i) + r_\infty$, with $i = 1$ or 2 . r_i are the anisotropies and θ_i are the respective rotational correlation times. The limiting anisotropies are: $r(t \rightarrow 0) = r_0$, and $r(t \rightarrow \infty) = r_\infty$, $r_0 = r_1 + r_2 + r_\infty$.

(a)

	Fluorescence lifetime						
	B_1	B_2	B_3	τ_1	τ_2	τ_3	τ^2
– Insulin	20.5	26.0	53.5	0.57	2.53	6.21	1.11
+ Insulin	21.2	30.8	48.0	0.49	2.29	6.15	1.08

(b)

	Anisotropy decay				
	r_0	r_1	r_∞	ϕ_1	χ^2
– Insulin	0.163	0.029	0.134	9.9	1.27
+ Insulin	0.16	0.059	0.101	59.3	1.06

steep decline levelling off at high anisotropy before the trace disappeared in the background noise. The value of 9.9 ns was higher than that usually measured in the case of amino acid side-chain mobility [38] and was much less than the value for the overall motion of the receptor (495 ns, calculated on the basis of a Stokes radius of 8 nm [39]). A detailed analysis was difficult due to the mismatch between overall correlation time, ϕ , and $\langle \tau \rangle$ [31] and because there was probably more than one domain involved in the 'inner' mobility of the receptor. Upon addition of insulin the correlation time rose to 59.3 ns, indicating a restriction of the inner mobility of the receptor.

DISCUSSION

Structural requirements for ectodomain function

The interaction of two $\alpha\beta$ half-receptors within the heterotetrameric ($\alpha\beta$)₂ holoreceptor complex is crucial for high-affinity insulin binding and co-operativity, tyrosine kinase activation and autophosphorylation [22–24], as well as insulin-inducible marked alterations in size and shape [22]. This implicates insulin-triggered extended conformational changes by which the polypeptide chains of fully functional heterotetrameric receptors are rearranged.

Although forming a heterotetrameric complex, HIR-ED or similar recombinant ectodomain constructs bind insulin with substantially lower affinity than wild-type receptors [26–28,34]. The relatively low free enthalpy of insulin binding may be due to

a reduced number of receptor–hormone interactions. Alternatively, low affinity could reflect a more rigid receptor structure that does not allow the energetically favourable conformational changes.

As shown for receptor species truncated immediately beyond the transmembrane anchors [34] and for our similar HIR-MAED construct, the presence of the membrane anchors is required and apparently sufficient for high-affinity hormone binding of heterotetrameric complexes. Accordingly, truncations at various sites further downstream have only a minor influence on insulin binding [33,34,40,41,41a].

Concomitant with a lowered affinity for the hormone, truncation immediately proximal to the hydrophobic membrane anchors leads to a water-soluble ectodomain receptor, HIR-ED, which subsequent to insulin binding is unable to undergo extended alterations in size and shape. As demonstrated for HIR-MAED species, the presence of the transmembrane anchors is not only critical for hormone binding but also confers additional wild-type-like properties to the ectodomain receptor moiety. This refers to insulin-inducible alterations in size and shape as well as to the different insertion properties of insulin-laden and free receptor complexes into artificial bilayer membranes from Triton X-114. Thus, the underlying molecular changes in the membrane-anchor-linked heterotetrameric receptor complexes occur within the ectodomain and require constraints that are brought about by the transmembrane anchors. In other words, the transmembrane anchors direct the extracellular polypeptide chains into the functional orientation that is required for both high-affinity insulin binding and insulin-inducible conformational alterations that lead to changes in size and shape.

Modulation of ectodomain function and signal propagation by transmembrane anchors

Many alterations within the amino acid sequence of the transmembrane anchors are critical for signal propagation from the extracellular to the cytoplasmic domains [42–44], proreceptor processing and receptor compartmentalization [45], or receptor down-regulation and internalization [46]. In contrast, high-affinity hormone binding of the ectodomain receptor moiety was retained in those receptor constructs and was only decreased with receptor species lacking the transmembrane helices [26–28,34]. Moreover, any pair of self-associating anchors within a heterotetrameric insulin–receptor complex is apparently sufficient to confer constraints to the ectodomain moieties that are required for normal hormone binding. This was demonstrated for insulin–receptor chimaerae in which the immunoglobulin constant domain [47], a leucine zipper [48] or the self-dimerizing transmembrane domain of glycophorin A [21] were exchanged for the transmembrane anchors. Interestingly, hormone-inducible changes in size and shape were also retained with an insulin receptor–glycophorin A construct [21]. Now, the present study demonstrates that marked alterations in size and shape no longer occur with receptor constructs lacking the transmembrane helices.

Models of structure and function

Although it does not bind hormone, the crystal structure of a polypeptide that embraces the two β -helical L-domains and the interconnecting cysteine-rich domain from the homologous insulin-like growth factor 1 receptor may also give a clue to initial conformational changes [49]. Since major hormone-binding sites have been found on all three domains [14–20], insulin is proposed to complex these domains in a horseshoe-like arrangement,

Figure 9 Possible mechanism of insulin-receptor activation

The elongated structure seen in most electron microscopic studies may resemble the most populated state of the insulin-free receptor. According to [55], in the insulin-bound state the transmembrane helices are mainly localized near the periphery of the globular receptor complex, and have been turned markedly towards the extracellular receptor moiety. This may also explain why insulin-laden full-length and HIR-MAED receptor conformers are hindered from being embedded into Triton X-114 membranes. The white ovals symbolize associated detergent.

burying itself in the cavity [49]. This bridging would restrict flexibility of the L1–Cys-rich fragment (where the L1 domain is apparently fixed to the Cys-rich domain by additional non-covalent interactions) towards the L2 domain and vice versa [49].

With respect to these hypotheses, fluorescence spectroscopy of the recombinant ectodomain construct [32], of the L1–Cys-rich–L2 fragment extended by the 16 C-terminal amino acid residues of the α -subunit [50] and of HIR-MAED has indicated a loss of flexibility when complexed with insulin. The spectroscopical properties may also reflect a similar initial binding process for all of the receptor forms studied. With respect to subsequent steps, CD and fluorescence spectra revealed similar secondary structures of both free and insulin-bound receptor species.

With respect to the proposed different sizes and shapes of insulin-bound and free receptor conformers, molecular electron microscopy should allow one to distinguish between these receptor forms. For full-length receptors, a variety of X- and, mainly, Y-like molecules, which are markedly elongated, has been observed, and a U- or V-shaped structure has been found for recombinant ectodomain species [32,51–54]. The results suggest a structure in which the two sets of the extracellular L1–Cys-rich fragments are splayed apart. In those studies, however, receptors could not be attributed to insulin-bound or free forms. Luo et al. [55] recently used gold-labelled insulin for visualizing distinct insulin-bound receptor species by cryo-electron microscopy. Surprisingly, detailed three-dimensional reconstruction revealed the spatial arrangement of subdomains within a globular complex in which the transmembrane anchors lie quite far apart from each other.

Thus logical conclusions on the basis of (i) recombinant receptors with artificial self-associating anchors, (ii) several electron microscopy studies and molecular modelling as well as (iii) direct experimental evidence obtained from different physicochemical properties of insulin-bound and free (full-length and HIR-MAED) receptor complexes imply that not only are certain subdomains involved but that global subdomain rearrangements

within the entire ectodomain moiety take place after insulin binding. As suggested in Figure 9, the proposed mechanism embraces the structural change from a markedly elongated insulin-free receptor complex to a globular, insulin-bound form. This model integrates lateral movements of the transmembrane helices, which are forced to the periphery of the receptor particle in the insulin-bound state. By this mechanism, the activation loop of one kinase domain would come into appropriate reach of the catalytic site of the opposing kinase domain and allows transphosphorylation of the critical tyrosine residues. Current studies in our laboratory with receptor constructs carrying green fluorescent protein should provide experimental access to assessment of variations in the distance between the transmembrane anchors.

Conclusions

Previously, heterotetrameric subunit organization has been demonstrated to be essential for insulin-inducible changes in physico-chemical properties. Now, (i) the underlying alterations have been attributed to structural changes within the ectodomain, rather than changes in secondary structure, (ii) a reorientation of subdomains is the reason for the different hydrodynamic properties of insulin-bound and free receptors, and (iii) the transmembrane anchors play a critical role in these changes. The results merge into a model that offers explanations for an extended understanding of insulin-receptor structure and function.

REFERENCES

- Ellis, L., Tavare, J. M. and Levine, B. A. (1991) Insulin receptor tyrosine kinase structure and function. *Biochem. Soc. Trans.* **19**, 426–432
- Lee, J. and Pilch, P. F. (1994) The insulin receptor: structure, function, and signaling. *Am. J. Physiol.* **266**, C319–C334
- Van Obberghen, E. (1994) Signalling through the insulin receptor and the insulin-like growth factor-I receptor. *Diabetologia* **37**(suppl. 2), S125–S134
- De Meyts, P. (1994) The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling. *Diabetologia* **37**(suppl. 2), S135–S148
- Taylor, S. S., Radzio-Andzelm, E. and Hunter, T. (1995) How do protein kinases discriminate between serine/threonine and tyrosine? Structural insights from the insulin receptor protein-tyrosine kinase. *FASEB J.* **9**, 1255–1266
- Hubbard, S. R. (1999) Structural analysis of receptor tyrosine kinases. *Progr. Biophys. Mol. Biol.* **71**, 343–358
- Marino-Buslje, C., Martin-Martinez, M., Mizuguchi, K., Siddle, K. and Blundell, T. L. (1999) The insulin receptor: from protein sequence to structure. *Biochem. Soc. Trans.* **27**, 715–726
- Adams, T. E., Epa, V. C., Garrett, T. P. and Ward, C. W. (2000) Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol. Life Sci.* **57**, 1050–1093
- Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203–212
- White, M. F. (1996) The IRS-signalling system in insulin and cytokine action. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **351**, 181–189
- Kadowaki, T., Tobe, K., Honda-Yamamoto, R., Tamemoto, H., Kaburagi, Y., Momomura, K., Ueki, K., Takahashi, Y., Yamauchi, T., Akanuma, Y. and Yazaki, Y. (1996) Signal transduction mechanism of insulin and insulin-like growth factor-1. *Endocr. J.* **43**(suppl.), S33–S41
- Schaffer, L. (1994) A model for insulin binding to the insulin receptor. *Eur. J. Biochem.* **221**, 1127–1132
- Bajaj, M., Waterfield, M. D., Schlessinger, J., Taylor, W. R. and Blundell, T. (1987) On the tertiary structure of the extracellular domains of the epidermal growth factor and insulin receptors. *Biochim. Biophys. Acta* **916**, 220–226
- Waugh, S. M., DiBella, E. E. and Pilch, P. F. (1989) Isolation of a proteolytically derived domain of the insulin receptor containing the major site of cross-linking/binding. *Biochemistry* **28**, 3448–3455
- Andersen, A. S., Kjeldsen, T., Wiberg, F. C., Vissing, H., Schaffer, L., Rasmussen, J. S., De Meyts, P. and Møller, N. P. (1992) Identification of determinants that confer ligand specificity on the insulin receptor. *J. Biol. Chem.* **267**, 13681–13686
- Fabry, M., Schaefer, E., Ellis, L., Kojro, E., Fahrenholz, F. and Brandenburg, D. (1992) Detection of a new hormone contact site within the insulin receptor ectodomain by the use of a novel photoreactive insulin. *J. Biol. Chem.* **267**, 8950–8956
- Schumacher, R., Soos, M. A., Schlessinger, J., Brandenburg, D., Siddle, K. and Ullrich, A. (1993) Signaling-competent receptor chimeras allow mapping of major insulin receptor binding domain determinants. *J. Biol. Chem.* **268**, 1087–1094
- Williams, P. F., Mynarcik, D. C., Yu, G. Q. and Whittaker, J. (1995) Mapping of an NH₂-terminal ligand binding site of the insulin receptor by alanine scanning mutagenesis. *J. Biol. Chem.* **270**, 3012–3016
- Mynarcik, D. C., Williams, P. F., Schaffer, L., Yu, G. Q. and Whittaker, J. (1997) Analog binding properties of insulin receptor mutants. Identification of amino acids interacting with the COOH terminus of the B-chain of the insulin molecule. *J. Biol. Chem.* **272**, 2077–2081
- Kristensen, C., Wiberg, F. C. and Andersen, A. S. (1999) Specificity of insulin and insulin-like growth factor I receptors investigated using chimeric mini-receptors. Role of C-terminal of receptor alpha subunit. *J. Biol. Chem.* **274**, 37351–37356
- Gardin, A., Auzan, C., Clauser, E., Malherbe, T., Aunis, D., Cremel, G. and Hubert, P. (1999) Substitution of the insulin receptor transmembrane domain with that of glycophorin A inhibits insulin action. *FASEB J.* **13**, 1347–1357
- Flörke, R. R., Klein, H. W. and Reinauer, H. (1990) Structural requirements for signal transduction of the insulin receptor. *Eur. J. Biochem.* **191**, 473–482
- Sweet, L. J., Morrison, B. D. and Pessin, J. E. (1987) Isolation of functional alpha beta heterodimers from the purified human placental alpha 2 beta 2 heterotetrameric insulin receptor complex. A structural basis for insulin binding heterogeneity. *J. Biol. Chem.* **262**, 6939–6942
- Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E. and Pilch, P. F. (1987) The insulin receptor. Structural basis for high affinity ligand binding. *J. Biol. Chem.* **262**, 8395–8401
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn, Cold Spring Harbor Press, Cold Spring Harbor, NY
- Ellis, L., Sissom, J. and Levitan, A. (1988) Truncation of the ectodomain of the human insulin receptor results in secretion of a soluble insulin binding protein from transfected CHO cells. *J. Mol. Recognit.* **1**, 25–31
- Johnson, J. D., Wong, M. L. and Rutter, W. J. (1988) Properties of the insulin receptor ectodomain. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7516–7520
- Whittaker, J. and Okamoto, A. (1988) Secretion of soluble functional insulin receptors by transfected NIH3T3 cells. *J. Biol. Chem.* **263**, 3063–3066
- Pilch, P. F. and Czech, M. P. (1980) The subunit structure of the high affinity insulin receptor. Evidence for a disulfide-linked receptor complex in fat cell and liver plasma membranes. *J. Biol. Chem.* **255**, 1722–1731
- Chen, G. E. and Yang, J. T. (1977) Hydrodynamic and optical properties of polyribonucleotides. *Anal. Lett.* **10**, 1195–1207
- Barkley, M. D., Kowalczyk, A. A. and Brand, L. (1981) Fluorescence decay studies of anisotropic rotations of small molecules. *J. Chem. Phys.* **75**, 3581–3593
- Schaefer, E. M., Erickson, H. P., Federwisch, M., Wollmer, A. and Ellis, L. (1992) Structural organization of the human insulin receptor ectodomain. *J. Biol. Chem.* **267**, 23393–23402
- Sasaoka, T., Takata, Y., Kusari, J., Anderson, C. M., Langlois, W. J. and Olefsky, J. M. (1993) Transmembrane signaling by an insulin receptor lacking a cytoplasmic beta-subunit domain. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4379–4383
- Whittaker, J., Garcia, P., Yu, G. Q. and Mynarcik, D. C. (1994) Transmembrane domain interactions are necessary for negative cooperativity of the insulin receptor. *Mol. Endocrinol.* **8**, 1521–1527
- Kopp, F. and Heusch, R. (1987) Membrane proteins in a lamellar phase of a polyglycol ether hydrate of Triton X-114. *Progr. Colloid Polymer Sci.* **73**, 146–149
- Flörke, R. R., Klein, H. W. and Reinauer, H. (1993) Differential insertion of insulin receptor complexes into Triton X-114 bilayer membranes. Evidence for a differential accessibility of the membrane-exposed receptor domain. *Eur. J. Biochem.* **211**, 241–247
- Lee, J., Pilch, P. F., Shoelson, S. E. and Scarlata, S. F. (1997) Conformational changes of the insulin receptor upon insulin binding and activation as monitored by fluorescence spectroscopy. *Biochemistry* **36**, 2701–2708
- Munro, I., Pecht, I. and Stryer, L. (1979) Subnanosecond motions of tryptophan residues in proteins. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 56–60
- Bucci, E. and Steiner, R. F. (1988) Anisotropy decay of fluorescence as an experimental approach to protein dynamics. *Biophys. Chem.* **30**, 199–224
- McClain, D. A., Maegawa, H., Levy, J., Huecksteadt, T., Dull, T. J., Lee, J., Ullrich, A. and Olefsky, J. M. (1988) Properties of a human insulin receptor with a COOH-terminal truncation. I. Insulin binding, autophosphorylation, and endocytosis. *J. Biol. Chem.* **263**, 8904–8911
- Haft, C. R. and Taylor, S. I. (1994) Deletion of 343 amino acids from the carboxyl terminus of the beta-subunit of the insulin receptor inhibits insulin signaling. *Biochemistry* **33**, 9143–9151
- Erratum (1995) *Biochemistry* **34**, 15880

- 42 Yamada, K., Goncalves, E., Kahn, C. R. and Shoelson, S. E. (1992) Substitution of the insulin receptor transmembrane domain with the c-neu/erbB2 transmembrane domain constitutively activates the insulin receptor kinase *in vitro*. *J. Biol. Chem.* **267**, 12452–12461
- 43 Li, S. C., Deber, C. M. and Shoelson, S. E. (1994) An irregularity in the transmembrane domain helix correlates with the rate of insulin receptor internalization. *Biochemistry* **33**, 14333–14338
- 44 Yamada, K., Carpentier, J. L., Cheatham, B., Goncalves, E., Shoelson, S. E. and Kahn, C. R. (1995) Role of the transmembrane domain and flanking amino acids in internalization and down-regulation of the insulin receptor. *J. Biol. Chem.* **270**, 3115–3122
- 45 Yamada, K., Goncalves, E., Carpentier, J. L., Kahn, C. R. and Shoelson, S. E. (1995) Transmembrane domain inversion blocks ER release and insulin receptor signaling. *Biochemistry* **34**, 946–954
- 46 Goncalves, E., Yamada, K., Thatte, H. S., Backer, J. M., Golan, D. E., Kahn, C. R. and Shoelson, S. E. (1993) Optimizing transmembrane domain helicity accelerates insulin receptor internalization and lateral mobility. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5762–5766
- 47 Bass, J., Kurose, T., Pashmforoush, M. and Steiner, D. F. (1996) Fusion of insulin receptor ectodomains to immunoglobulin constant domains reproduces high-affinity insulin binding *in vitro*. *J. Biol. Chem.* **271**, 19367–19375
- 48 Hoyne, P. A., Cosgrove, L. J., McKern, N. M., Bentley, J. D., Ivancic, N., Elleman, T. C. and Ward, C. W. (2000) High affinity insulin binding by soluble insulin receptor extracellular domain fused to a leucine zipper. *FEBS Lett.* **479**, 15–18
- 49 Garrett, T. P., McKern, N. M., Lou, M., Frenkel, M. J., Bentley, J. D., Lovrecz, G. O., Elleman, T. C., Cosgrove, L. J. and Ward, C. W. (1998) Crystal structure of the first three domains of the type-1 insulin-like growth factor receptor. *Nature (London)* **394**, 395–399
- 50 Schlein, M., Havelund, S., Kristensen, C., Dunn, M. F. and Kaarsholm, N. C. (2000) Ligand-induced conformational change in the minimized insulin receptor. *J. Mol. Biol.* **303**, 161–169
- 51 Christiansen, K., Trandum-Jensen, J., Carlsen, J. and Vinten, J. (1991) A model for the quaternary structure of human placental insulin receptor deduced from electron microscopy. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 249–252
- 52 Trandum-Jensen, J., Christiansen, K., Carlsen, J., Brenzel, G. and Vinten, J. (1994) Membrane topology of insulin receptors reconstituted into lipid vesicles. *J. Membr. Biol.* **140**, 215–223
- 53 Woldin, C. N., Hing, F. S., Lee, J., Pilch, P. F. and Shipley, G. G. (1999) Structural studies of the detergent-solubilized and vesicle-reconstituted insulin receptor. *J. Biol. Chem.* **274**, 34981–34992
- 54 Tulloch, P. A., Lawrence, L. J., McKern, N. M., Robinson, C. P., Bentley, J. D., Cosgrove, L., Ivancic, N., Lovrecz, G. O., Siddle, K. and Ward, C. W. (1999) Single-molecule imaging of human insulin receptor ectodomain and its Fab complexes. *J. Struct. Biol.* **125**, 11–18
- 55 Luo, R. Z., Beniac, D. R., Fernandes, A., Yip, C. C. and Ottensmeyer, F. P. (1999) Quaternary structure of the insulin-insulin receptor complex. *Science* **285**, 1077–1080

Received 1 June 2001/16 August 2001; accepted 12 September 2001