Oligomeric States of the Insulin Receptor: Binding and Autophosphorylation Properties†

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ABSTRACT: Properties of oligomeric states of the insulin receptor were analyzed by polyacrylamide gel electrophoresis in nondenaturing buffer conditions (ND-PAGE). Partially purified insulin receptors resolve in ND-PAGE as three distinct species: (i) the fast electrophoretic mobility, low molecular mass form manifests intense labeling by iodinated insulin and shows basal and insulin-stimulated autophosphorylation; (ii) the middle, intermediate mobility form exhibits strong labeling by iodinated ligand but does not possess the capacity to be autophosphorylated; (iii) the slow mobility, highest molecular mass form necessitates covalent binding with iodinated hormone to withstand electrophoresis and shows autophosphorylation enhanced by insulin. This receptor form is more heavily labeled by phosphorylation than the low form. At 22 °C, binding and autophosphorylation do not appear to be time dependent. At 37 °C, binding and autophosphorylation of low and high species attain a maximum after 15 min and then decrease as time of incubation with insulin is prolonged to 120 min; the middle species exhibits a much slower association rate, and its labeling by iodinated hormone becomes more intense with time. Our data show that in cell-free systems insulin receptors appear in various oligomeric states and that the highest molecular mass oligomer exhibits the most pronounced autophosphorylation. This is compatible with the concept that insulin receptor oligomerization provides a mechanism for transmembrane signaling.

Elucidation of insulin-induced transmembrane signaling remains a challenging problem in spite of important developments accomplished in recent years. Insulin binds to the extracellular α subunit of its specific $\alpha_2\beta_2$ receptor whose linear amino acid structure is now known (Ebina et al., 1985; Ullrich et al., 1985). After the binding event, the cytoplasmic domain of the transmembrane β subunit undergoes insulin-induced receptor kinase activation and autophosphorylation (Kasuga et al., 1982a,b,c; Van Obberghen & Kowalski, 1982; Van Obberghen et al., 1983). Receptor kinase activation has been shown to be essential for generation of insulin's metabolic (Chou et al., 1987; Ebina et al., 1987; Morgan & Roth, 1987) and growth promoting effects (Chou et al., 1987). Recent studies on cooperation between $\alpha\beta$ receptor halves (Böni-Schnetzler et al., 1986, 1987; Sweet et al., 1987a,b) stressed the critical importance of communication between two $\alpha\beta$ complexes within the $\alpha_2\beta_2$ receptor for attaining high affinity for insulin and sensitive insulin-stimulated kinase activity.

Oligomerization of insulin receptor molecules is thought to be involved in hormonal signal transmission across the cell membrane. The receptor aggregation phenomenon has been observed on intact cell surfaces (Jarrett et al., 1974; Orci et al., 1975) and in purified receptor preparations (Crettaz et al., 1984; Chen et al., 1986). The curvilinearity of a Scatchard plot of insulin binding displayed by insulin receptors together with findings of only one binding site on the $\alpha_2\beta_2$ receptor points to the possible existence of oligomeric receptor forms (Sweet et al., 1987a). Cross-linking of insulin receptors or of insulin receptor domains within a receptor oligomer has been shown to be necessary for activation of the solubilized receptor kinase (Heffetz & Zick, 1986) and for initiation of insulin-like biological effects evoked by antibodies to insulin receptor (Kahn et al., 1978). What might appear at variance with these observations are "receptor dilution" experiments (Shia et al., 1983; Petruzzelli et al., 1984) and analysis of phospholipid vesicles containing a single insulin receptor (Sweet et al., 1985), which indicated that insulin-stimulated kinase activity and consequent β subunit autophosphorylation seem to be intramolecular processes, and thus not requiring receptor–receptor interaction.

To investigate further the conditions in which the putative oligomeric states of insulin receptor could occur, we analyzed here the electrophoretic mobility of solubilized receptors under nondissociating buffer conditions. This technique, which proved fruitful in analysis of ligand-induced dimerization of EGF[1] receptors (Yarden & Schlessinger, 1987), is designed to fractionate a protein mixture in such a way that subunit interaction and native protein conformations are preserved. We found that in nondissociating buffer conditions solubilized, partially purified, insulin receptors migrate as three distinct molecular species, exhibiting dissimilar insulin binding and autophosphorylation properties.

Materials and Methods

Materials. Porcine insulin was a gift from Novo Research Institute (Copenhagen, Denmark). Photoreactive insulin analogue B2-Napa insulin was a gift from Dr. D. Brandenburg (Aachen, FRG). All reagents for ND-PAGE and SDS–PAGE were from Bio-Rad Laboratories (Richmond, CA), except for the high molecular mass marker proteins which were from Pharmacia. Radioactive materials were purchased from

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[1] Abbreviations: ND-PAGE, nondenaturing polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; HSA, human serum albumin; WGA, wheat germ agglutinin; EGF, epidermal growth factor; kDa, kilodalton(s).

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Amersham. The chloramine-T method was used for the iodination of native and photoreactive B2-Napa insulin to a specific activity of 200–250 Ci/g (Fehlmann et al., 1982).

Cells. Rat hepatoma cells (Fao) were cultured in F12 medium supplemented with 10% fetal calf serum (FCS). Rat fibroblasts expressing human insulin receptor (RHIR) were a gift from Dr. Axel Ullrich (Genentech, South San Francisco, CA). They were cultured in H21/F12 medium (1:1) supplemented with 7% dialyzed FCS and 5×10^{-7} M methotrexate. Cells were grown to confluency in 175-cm^2 plastic dishes (Falcon).

Partial Purification of Insulin Receptors. Cells were washed once with 50 mM Hepes (pH 7.6)–150 mM NaCl solution and then detached from the plastic dish and solubilized by continuous stirring for 1 h at 4 °C with buffer containing 50 mM Hepes (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM bacitracin, aprotinin (1000 trypsin inhibitor units/mL), and 1 mM PMSF. The soluble fraction obtained upon centrifugation at 100000*g* for 1 h at 4 °C was applied to a WGA–agarose column and recycled three times. After being washed with 30 mM Hepes (pH 7.6), 30 mM NaCl, and 0.1% Triton X-100 (HNT) buffer, bound glycoproteins were desorbed from the column with 0.3 M *N*-acetyl-D-glucosamine. The eluted glycoproteins were stored in aliquots at −80 °C. In the experiments depicted in Figures 3, 5, 6, and 8 the solubilization buffer used was 50 mM Hepes (pH 7.6), 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, and 1% Triton X-100 (supplemented with protease inhibitors as above), and HNT buffer was replaced by 50 mM Hepes (pH 7.6), 10% glycerol, and 0.1% Triton X-100 buffer.

Nondenaturing Polyacrylamide Gel Electrophoresis (ND-PAGE). ND-PAGE was carried out essentially as described by Yarden and Schlessinger (1987). Briefly, acrylamide and bis(acrylamide) dissolved in electrophoresis buffer [90 mM tris(hydroxymethyl)aminomethane, 80 mM boric acid, 2.5 mM EDTA, 0.3% Triton X-100 (pH 8.4)] were used at a ratio 24:1. A linear gradient of 2%–15% of acrylamide, without a stacking gel, was used. Sample buffer was prepared with 2× concentrated electrophoresis buffer containing 27% sucrose and 0.01% bromophenol blue; 200 mM NaF and 40 mM EDTA were added when the receptors were labeled with ^{32}P. Gels were equilibrated for 20 min by electrophoresis at 4 °C at 70 V; then, the samples, mixed (1:1) with sample buffer, were loaded and electrophoresed at 4 °C, first at 70 V for 20 min and then at 150 V for 16–18 h. The high molecular mass calibration kit from Pharmacia (thyroglobulin monomer, 670 kDa; thyroglobulin dimer, 1340 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; albumin, 67 kDa) was used as a reference and to allow comparison between experiments. Note that in native gels molecular mass markers can be used only to assign individual bands and cannot be considered as molecular mass determinations. Staining, destaining, drying, and autoradiography of ND gels were carried out according to standard procedures.

Binding of Radiolabeled Insulin to Its Receptor. Partially purified insulin receptors (15 μL of WGA column eluate, corresponding to approximately 7 μg of glycoproteins) were incubated with 10 μL of iodinated insulin at a final concentration of 10^{-7} M corresponding to 2×10^6 cpm. Nonspecific binding was determined by adding unlabeled insulin (3×10^{-5} M) together with labeled insulin. In experiments with ^{125}I-B2-Napa insulin, the incubation was performed in the dark; thereafter, the samples were irradiated for 5 min with ultraviolet light (Fehlmann et al., 1982). For each experiment the incubation durations and temperatures are indicated in the legends to the figures. After binding, samples were mixed (1:1) with sample buffer and subjected to ND electrophoresis.

Autophosphorylation of Insulin Receptors. Receptor preparations (15 μL) were incubated for 45 min without or with insulin in 30 mM Hepes (pH 7.6), 30 mM NaCl, and 0.1% Triton X-100 (HNT) buffer. Final insulin concentrations and incubation temperature are indicated in the figure legends. Phosphorylation was initiated by adding 5 μL of reaction mix at final concentrations of 15 μM [γ-^{32}P]ATP, 4 mM $MnCl_2$, and 8 mM $MgCl_2$ and carried out for 20 min at 22 °C. Reactions were terminated by adding sample buffer (1:1) supplemented with 200 mM NaF and 40 mM EDTA, and finally, the samples were subjected to ND-PAGE.

ND-PAGE Followed by SDS-PAGE. Receptor preparations (60 μL) were incubated with insulin, autophosphorylated, and analyzed by ND gel electrophoresis as described above. The wet nonfixed gel was autoradiographied overnight at −80 °C. The labeled bands identified by autoradiography were excised from the frozen gel and soaked in 400 μL of a solution containing 50 mM Tris, 3% SDS, and 10% glycerol, pH 6.8. After 16 h at 30 °C, the eluted radioactivity, which represented about 50% of the radioactivity contained in the excised bands, was loaded on a 5% polyacrylamide–SDS gel and submitted to electrophoresis in nonreducing conditions. The molecular masses of the marker proteins were as follows: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 93 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa. Staining, destaining, drying, and autoradiography were carried out according to standard procedures.

Results

Insulin Receptor Species Labeled with ^{125}I-Insulin. To investigate the possible existence of oligomeric insulin receptor states without the use of cross-linking agents, ^{125}I-insulin was bound to receptor, and hormone–receptor complexes were then analyzed by nondenaturing gel electrophoresis. In brief, insulin receptor preparations from Fao cells were incubated with iodinated insulin for 15, 60, and 120 min at 22 and 37 °C and then subjected to ND-PAGE. As illustrated in Figure 1 (lanes A–G), two specific bands were visualized at 580 and 950 kDa, respectively, when compared with calibration markers. The 380-kDa band and 160-kDa band were considered not specific, since they were not displaced by a 100-fold excess of unlabeled insulin (lane G); they will not be discussed any longer. At 22 °C the intensity of the 580-kDa band remained the same for all incubation times, whereas the intensity of the 950-kDa band was slightly lower after 15 min than after 60 and 120 min of incubation (lanes A, C, and E). At 37 °C the intensity of the 580-kDa band decreased while the intensity of the 950-kDa band increased as the incubation time was prolonged, from 15 to 120 min with the major increment occurring between 15 and 60 min (lanes B, D, and E). Thus, at time points 60 and 120 min, the 580-kDa receptor form was less labeled by iodinated insulin at 37 °C than at 22 °C, while the 950-kDa form was more heavily labeled.

To exclude the possibility that these observations were inherent to Fao insulin receptors, we next extended our studies to receptors from another source, i.e., receptors obtained from RHIR cells. Three labeled bands were detected as demonstrated in Figure 2. In addition to the molecular species found with solubilized Fao cell receptors, 580- and 950-kDa forms, a third receptor form, localized at 1200 kDa, could be detected. Its very weak labeling by ^{125}I-insulin may be due to (i) low affinity for the ligand or high dissociation rate, which electrophoresis could further decrease or increase, respectively, and/or (ii) a small quantity of this species present in the WGA

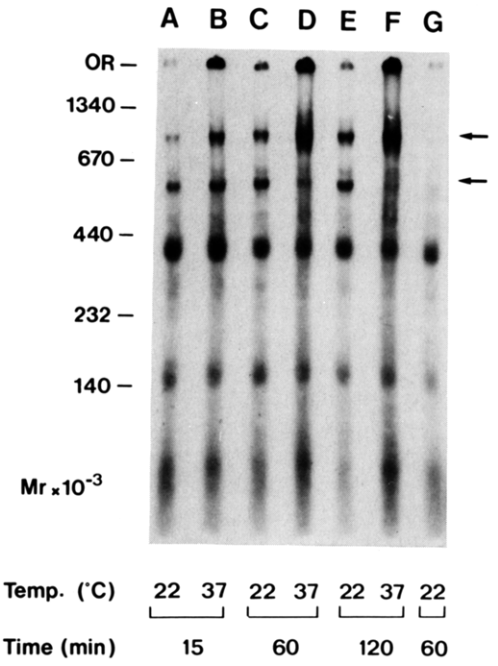

FIGURE 1: Insulin receptors labeled with ^{125}I-insulin: analysis by ND-PAGE. Partially purified insulin receptors from Fao cells (15 μL of the WGA column eluate, corresponding to approximately 7 μg of glycoproteins) were incubated with 10 μL of ^{125}I-insulin at a final concentration of 10^{-7} M (2×10^6 cpm) for the indicated time periods and temperatures. Nonspecific binding was determined by adding insulin (3×10^{-5} M) together with labeled insulin (lane G). The samples were analyzed by ND-PAGE as described under Materials and Methods, and an autoradiogram (14-h exposure) of the gel is shown.

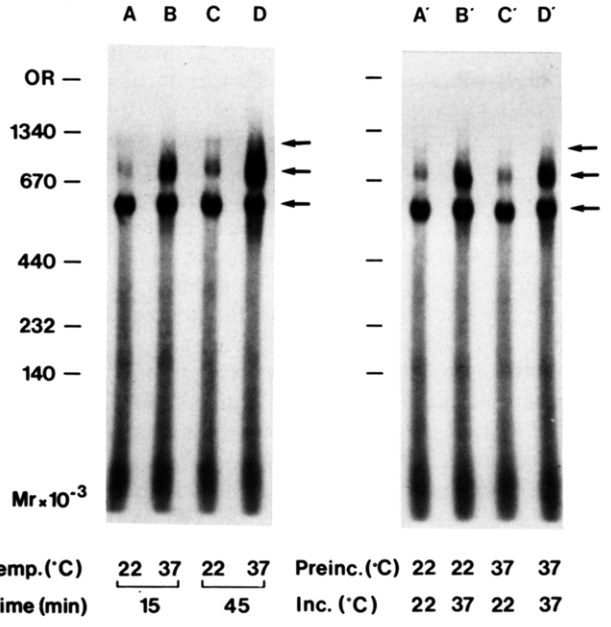

FIGURE 2: Time and temperature dependence of insulin receptor labeling with iodinated insulin. (Left panel) Partially purified receptor preparations from RHIR cells (10 μL) were incubated with 10 μL of ^{125}I-insulin (10^{-7} M, about 2×10^6 cpm) for the indicated time periods and temperatures. (Right panel) Partially purified receptor preparations from RHIR cells (10 μL) were first preincubated (preinc.) for 30 min at 22 °C (lanes A′ and B′) or 37 °C (lanes C′ and D′) and then incubated (inc.) with 10 μL of ^{125}I-insulin for 15 min at 22 °C (lanes A′ and C′) or 37 °C (lanes B′ and D′). The samples were analyzed by ND-PAGE as described under Materials and Methods and an autoradiogram (2-h exposure) of the gel is shown.

preparation, and/or (iii) a relative inaccessibility of the reactive site(s). These mechanisms could occur independently or combined.

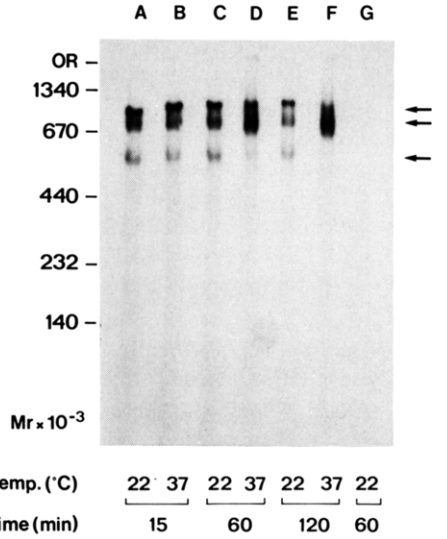

FIGURE 3: Insulin receptors labeled with photoreactive B2-Napa ^{125}I-insulin: analysis by ND-PAGE. Partially purified insulin receptors from RHIR cells (15 μL) were incubated in the dark with 5 μL of photoreactive B2-Napa ^{125}I-insulin at a final concentration of 50 nM, corresponding to 1×10^6 cpm, for the indicated time periods and temperatures and ultraviolet irradiated. Nonspecific binding was determined by adding insulin (3×10^{-5} M) together with labeled insulin (lane G). The samples were analyzed by ND-PAGE as described under Materials and Methods, and an autoradiogram (2-h exposure) of the gel is shown.

At 22 °C (lanes A and C) the high, 1200-kDa, band was weakly labeled, the middle, 950-kDa, band was more labeled, and their intensities increased with length of incubation. The intensity of the low, 580-kDa, band was the most important, and stable with time (lanes A and C). At 37 °C (lanes B and D) the 1200-kDa band disappeared and the 950-kDa band became the most strongly labeled, and even more so as incubation time increased, while the low band decreased slightly [this effect is seen on an underexposed film (see also Figure 7)].

In an attempt to discriminate between the effects of temperature and insulin alone on the appearance of receptor species, the receptor preparations were first preincubated for 30 min at 22 or 37 °C and then incubated with ^{125}I-insulin for 15 min at 22 or 37 °C in such a way that all temperature combinations (22 and 37 °C) were tested. It was found that incubation of receptor preparations with insulin at 37 °C resulted in increased labeling of the middle band for both preincubation conditions (Figure 2, lanes B′ and D′). In contrast, a preincubation of receptors at 37 °C followed by an incubation with ligand at 22 °C did not prove to be sufficient to induce the effect (Figure 2, lane C′). A possible conversion from low to middle form induced by temperature alone and then reversal during 15 min of subsequent incubation at 22 °C could occur, but it should not be compared to an effect at 37 °C which withstands overnight electrophoresis at 4 °C.

To further investigate the nature of ligand interactions with the separate receptor species, covalent cross-linking of iodinated, photoreactive B2-Napa insulin to receptors from RHIR cells was analyzed. Comparing Figures 1 and 2 with Figure 3 confirmed the time and temperature dependence of labeling of low, middle, and high species. Interestingly, the high receptor form appeared to necessitate covalent affinity binding to withstand electrophoresis, pointing to its weaker interaction with insulin.

To explain the distinct labeling of receptor species, at least the three following possibilities can be envisaged. First, the

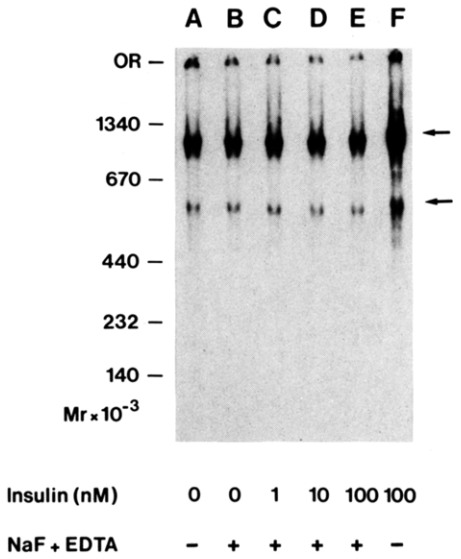

FIGURE 4: Insulin receptors labeled with ^{32}P: analysis by ND-PAGE. Receptor preparations from RHIR cells (10 μL) were phosphorylated for 45 min in the absence of insulin as described under Materials and Methods. Phosphorylated samples were then incubated for 1 h at 37 °C without (lanes A and B) or with (lanes C–F) increasing concentrations of insulin as indicated. The samples corresponding to lanes B–E were treated with NaF (100 mM) and EDTA (20 mM) prior to incubation with insulin. The samples were analyzed by ND-PAGE as described under Materials and Methods, and an autoradiogram (2-h exposure) of the gel is shown.

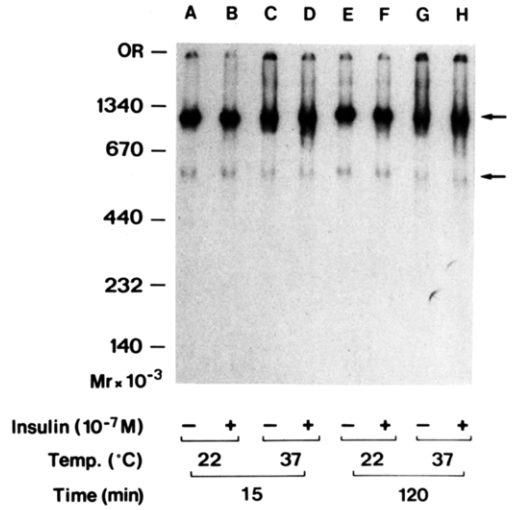

FIGURE 5: Insulin receptors labeled with ^{32}P: time and temperature dependence of response to insulin. Insulin receptors autophosphorylated as described in the legend to Figure 4 were incubated without or with insulin (10^{-7} M) for the indicated time periods and temperatures. Samples were then resolved by ND-PAGE, and the resulting autoradiogram (2-h exposure) of the gel is shown.

different labeling could be due to the fact that incubation of receptors with ligand at higher temperature creates a more stable, less reversible interaction, which better resists electrophoresis. However, if this were the case, both bands should be more labeled at 37 °C than at 22 °C. A second possibility would be that different molecular species are labeled with different kinetics at 37 °C; i.e., the 1200- and 580-kDa forms would exhibit fast association rates and attain maximum binding after 15 min of incubation and then dissociate, while the 950-kDa form could manifest a slower association rate and require 60 min to attain plateau binding. The third possibility would be a conversion of the 580- and 1200-kDa bands to the 950-kDa band under the effect of insulin at 37 °C.

These data show that the observed appearance of the receptor species depends on both temperature and hormone. However, labeling with insulin itself, which acts at the same time as a possible modulator of receptor forms and as a tagging device, does not permit determination of whether the described characteristics are due to binding properties or to a possible hormone-induced conversion.

Basal and Insulin-Induced Autophosphorylation of Insulin Receptor Species. We next investigated the possible effect of phosphorylation on the appearance of the different receptor species. First, we carried out basal autophosphorylation of RHIR receptor preparations for 45 min, and then we incubated the phosphoproteins without (Figure 4, lanes A and B) or with increasing insulin concentrations (lanes C–F) for 1 h at 37 °C (Figure 4) and 22 °C (data not shown). As illustrated in Figure 4, two phosphoproteins were detected with an apparent molecular mass of 1200 and 580 kDa, respectively, which are similar to the bands visualized with ^{125}I-insulin. In contrast, the 950-kDa band was not seen by ^{32}P labeling. When NaF (100 mM) and EDTA (20 mM) were omitted from the samples, no dephosphorylation could be detected as illustrated by the comparison of lane A to lane B (both without insulin). Further, comparison of lane E to control lane F (both with 10^{-7} M insulin) showed that without ion chelation receptor kinase activity persisted in the presence of insulin and autophosphorylation of both forms increased.

In a second series of experiments receptors were again phosphorylated in the absence of insulin. After termination of their phosphorylation, they were subsequently incubated with insulin (10^{-7} M) for shorter (15 min) or longer (120 min) durations at 22 and 37 °C (Figure 5). Incubation with hormone did not modify the labeling pattern, illustrating again the absence of the middle form and the lack of apparent interconversion between the low and high species at least within the time frame studied. These findings apply as well to receptor preparations which were not previously submitted to storage at −80 °C. Further, we found exactly the same behavior of phosphorylated insulin receptors, when receptors were prepared in the absence (Figure 5) or the presence (Figure 4) of NaCl and the absence (Figure 4) or the presence (Figure 5) of 10% glycerol, which could have influenced an insulin-induced conversion.

Next, we analyzed the insulin-stimulated kinase activity of the receptor forms. Receptor preparations from Fao cells were incubated without or with increasing insulin concentrations for 45 min at 22 or 37 °C and then phosphorylated with [γ-^{32}P]ATP for 20 min at 22 °C as described under Materials and Methods. Two receptor states were detected on the autoradiogram (data not shown). The high, 1200-kDa receptor form was discernible in the absence of insulin, and its autophosphorylation was strongly stimulated by insulin in a dose-dependent manner. For both basal and insulin-stimulated phosphorylation the low, 580-kDa band was less heavily labeled when compared to the high receptor form. This moderate insulin-stimulated receptor autophosphorylation could have been due to serine phosphorylation with subsequent inhibition of the receptor tyrosine kinase (Yu et al., 1985). However, alkaline treatment of the gels indicated that this appears not to be the case (data not shown). Similar to what we found in Figure 4, the middle, 950-kDa form of the receptor was not visualized, even on an overexposed film. It seems thus that this insulin receptor species does not appear in a phosphorylated state. Incubation of receptor preparation with insulin at 37 °C compared to 22 °C did not change the general pattern of autophosphorylation, but basal and insulin-stimulated autophosphorylation were less pronounced at 37 °C than at 22 °C. Further, the appearance of the two phosphorylated forms

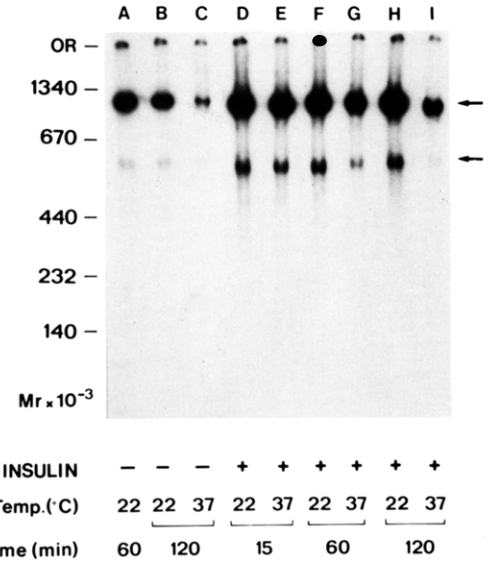

FIGURE 6: Time and temperature dependence of insulin-induced receptor phosphorylation. Receptor preparations from RHIR cells (10 μL) were incubated without (lanes A–C) or with (lanes D–I) 10^{-7} M insulin for the indicated time periods and temperatures and then phosphorylated with [γ-^{32}P]ATP for 20 min at 22 °C and analyzed by ND-PAGE as described under Materials and Methods. The autoradiogram (4-h exposure) of the gel is shown.

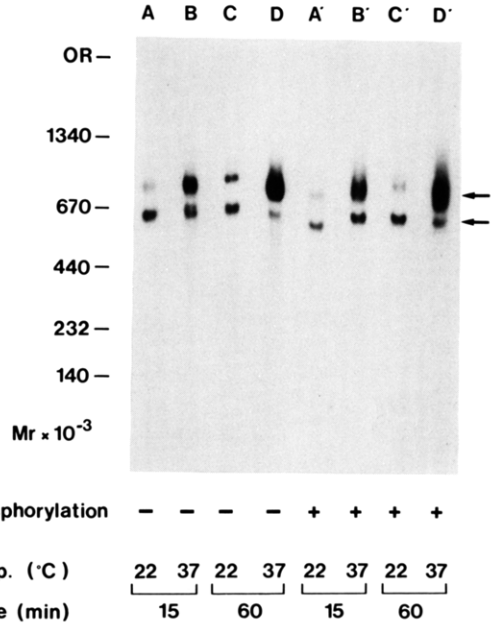

FIGURE 7: Lack of effect of phosphorylation on hormone binding. Receptor preparations from RHIR cells (10 μL) were incubated without (lanes A–D) or with (lanes A′–D′) the nonradioactive phosphorylation mix (15 μM ATP, 4 mM $MnCl_2$, 8 mM $MgCl_2$) for 1 h at 22 °C. Then, the reaction was stopped by the addition of NaF (100 mM) and EDTA (20 mM), and samples were incubated with iodinated insulin for 15 and 60 min at 22 and 37 °C as indicated. The autoradiogram (2-h exposure) of the ND gel is shown.

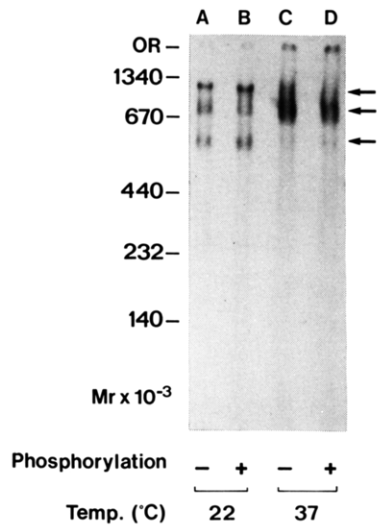

FIGURE 8: Lack of effect of phosphorylation on receptor–photoreactive ^{125}I-insulin complexes. Insulin receptors were treated as described in the legend to Figure 3 for 120 min at 22 and 37 °C. Aliquots were then incubated for 20 min at 22 °C with (lanes B and D) or without (lanes A and C) nonradioactive solution (15 μM ATP, 4 mM $MnCl_2$, 8 mM $MgCl_2$). Samples were resolved by ND-PAGE, and the resulting autoradiogram (2-h exposure) of the gel is shown.

was not affected by receptor concentration, the ratio of cpm in high form to cpm in high + low forms remaining at a constant value of approximately 0.8 (data not shown). Finally, the cation (Mn^{2+}/Mg^{2+}) requirements for autophosphorylation of the high and low forms were the same, and there appeared to be no detectable cation-independent autophosphorylation (data not shown).

Time and temperature dependence of insulin-stimulated receptor autophosphorylation was next explored. Receptor preparations from RHIR cells were incubated for 15, 60, and 120 min with insulin (10^{-7} M) at 22 and 37 °C and then phosphorylated for 20 min at 22 °C and submitted to ND-PAGE. As illustrated in Figure 6, insulin-induced autophosphorylation of low and high forms decreased as the duration of stimulation by hormone at 37 °C was prolonged (lanes E, G, and I) and was not modified by the incubation duration at 22 °C (lanes D, F, and H). Basal autophosphorylation appeared weaker at 37 °C than at 22 °C at 120 min (Figure 6, lanes B and C) and 60 min (data not shown). These results can be interpreted in at least two ways: either low and high forms progressively disappeared as the incubation time at 37 °C increased (however this is not supported by data shown in Figures 4 and 5), or incubation at 37 °C led to changes of low and high forms such that hormone binding (Figures 1–3) and basal and ligand-stimulated autophosphorylation were diminished.

Effect of Phosphorylation on Appearance of Receptor Species. Here we investigated whether the "disappearance" of the 950-kDa receptor form was linked to a modification related to phosphorylation. In a first set of experiments we incubated receptor preparations without (Figure 7, lanes A–D) or with (Figure 7, lanes A′–D′) the nonradioactive phosphorylation mix for 1 h at 22 °C. The reaction was stopped by ion chelation, and samples were incubated with iodinated insulin for 15 and 60 min at 22 and 37 °C. As shown, receptor phosphorylation did not modify insulin binding to low, middle, and high receptor species.

In a second series of experiments we explored whether phosphorylation of insulin–receptor complexes could modify their aggregation state. To this end receptor preparations were incubated with B2-Napa insulin at 22 and 37 °C for 120 min (Figure 8) and 60 min (data not shown); after covalent ligand attachment, incubation without (lanes A and C) or with (lanes B and D) the nonradioactive ATP mix was carried out for 20 min at 22 °C. As demonstrated, phosphorylation of receptor–hormone complexes did not influence the occurrence of low, middle, and high species.

There appears to exist a clear uncoupling between the labeling pattern with iodinated hormone and that seen with ^{32}P. It is possible that the middle form arose from the high form as an "artifact" due to different buffer conditions. To rule this

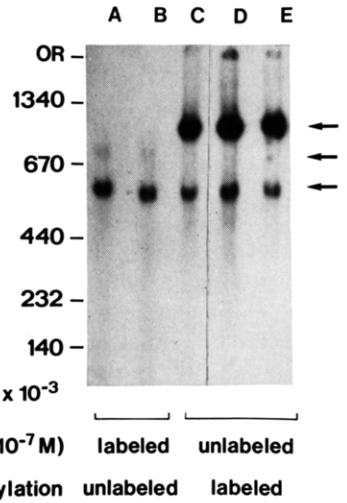

FIGURE 9: Uncoupling between hormone binding and autophosphorylation properties of insulin receptor species. Receptor preparations from RHIR cells (10 μL) were incubated with radiolabeled (lanes A and B) or unlabeled (lanes C–E) insulin (10^{-7} M) for 45 min at 22 °C and then phosphorylated with unlabeled (lane B) or radioactive (lanes C–E) ATP as described under Materials and Methods. Incubation with insulin (lanes A–D) and phosphorylation of receptor preparations (lanes B–D) were carried out in the same solution containing 0.2 M glycine and 0.25% human serum albumin (HSA) at pH 8.8 (lanes A–C) or pH 7.4 (lane D). The sample in lane E was treated in HNT buffer as in the previous phosphorylation experiments. The autoradiogram (6-h exposure) of the ND gel is shown.

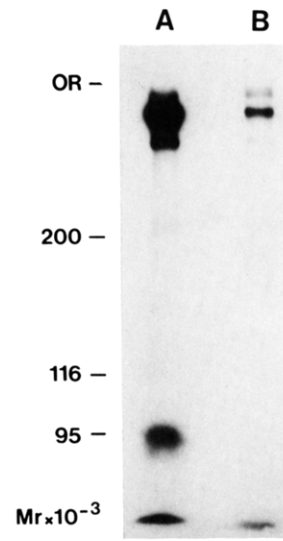

FIGURE 10: SDS–PAGE of phosphorylated insulin receptors eluted from ND-PAGE. Receptor preparations from Fao cells (60 μL) incubated with insulin, autophosphorylated, and analyzed by ND gel electrophoresis were excised and eluted from the ND gel as described under Materials and Methods. The radioactive phosphoproteins eluted from the high, 1200-kDa band (lane A) and from the low, 580-kDa band (lane B) were loaded on a 5% polyacrylamide–SDS gel and submitted to electrophoresis in nonreducing conditions.

out, samples were concomitantly incubated with radiolabeled (Figure 9, lanes A and B) or unlabeled insulin (lanes C–E) and then phosphorylated with unlabeled (lane B) or radioactive ATP (lanes C–E). Incubation with insulin (lanes A–D) and subsequent phosphorylation of receptor preparations (lanes B–D) were carried out in the same 200 mM glycine/0.25% HSA solution at pH 8.8 (lanes A–C) or at pH 7.4 (lane D), while the sample in lane E was processed in HNT buffer as in previous autophosphorylation experiments. The appearence of high, middle, and low insulin receptors species was stable in the range of tested ionic strength, detergent concentrations, and pH.

Analysis of Oligomeric State of Receptor Species. To investigate whether any of the receptor forms identified in our previous sections may represent oligomeric insulin receptors, labeled phosphoproteins eluted from the nondenaturing gel were submitted to SDS–PAGE as described under Materials and Methods. Our working hypothesis was that, if a labeled band did contain the $\alpha_2\beta_2$ receptor form, the band with higher molecular mass was likely to represent an oligomeric $(\alpha_2\beta_2)_n$ state. Figure 10 demonstrates that the phosphoproteins eluted not only from the high, 1200-kDa band (lane A) but also from the low, 580-kDa band (lane B) migrated as a doublet at ~350–380 kDa on the SDS–polyacrylamide gel in nonreducing conditions. The same observations were made with high and middle receptor species labeled with B2-Napa insulin, which also migrated at ~350–380 kDa on SDS–PAGE (data not shown). Notice that the differences between the intensities of the labeled spots in lanes A and B were due to different amounts of radioactive material loaded on the SDS gel and did not allow any quantification of the low and high receptor species. The lower part of the doublet seen in Figure 10 could represent an $\alpha_2\beta$ receptor or an $\alpha_2\beta'_2$ receptor where the β subunits have been clipped to β' forms. To discriminate between these possibilities we have performed a reduced gel equivalent to lane A and found all the counts in the 95-kDa band (data not shown). In addition, analysis of the receptor directly on SDS–PAGE showed the simultaneous presence of free receptor β subunits and the lower part of the doublet (data not shown). As a whole we interpret these data to mean that the lower part of the doublet seen in Figure 10 corresponds to an $\alpha_2\beta$ receptor rather than a $\alpha_2\beta'_2$ receptor with clipped β' subunits.

The nature of the three distinct insulin receptor species resolved by the native gel system (Figures 1–4) is an important question, but a difficult one to answer given the very limited value of molecular mass determinations especially in case of large hydrophobic glycoproteins such as the insulin receptor. In an attempt to address this issue, insulin receptors were denatured by heating for 5 min at 95 °C in the presence of 1% SDS and DTT (2 and 20 mM). The denatured receptors were then analyzed by native gels. With phosphorylated receptors three phosphoproteins were found with the following apparent molecular masses: 150, 400, and 600 kDa (data not shown). These phosphoproteins correspond very likely to receptor β subunits, $\alpha\beta$ receptor halves or proreceptors, and $\alpha_2\beta_2$ receptor monomers, respectively. Similar analysis of receptors labeled with photoreactive ^{125}I-insulin gave three labeled bands with apparent molecular masses of 230, 400, and 600 kDa (data not shown). We recognize these proteins as α subunits, $\alpha\beta$ receptor halves or proreceptors, and $\alpha_2\beta_2$ monomers. Taken together our data would indicate that the low molecular mass receptor species seen in native gel can be identified as the receptor $\alpha_2\beta_2$ monomer. The medium and high molecular mass species likely correspond to oligomers of $\alpha_2\beta_2$ with the same composition but with different electrophoretic behavior; alternatively, the medium species could be a dimer, $(\alpha_2\beta_2)_2$, and the high one an oligomer, $(\alpha_2\beta_2)_n$ with $n > 2$.

Discussion

To investigate oligomerization of insulin receptors and its possible control mechanism, we analyzed the electrophoretic mobility of solubilized receptors under nondissociating buffer conditions. Using this approach we found that partially purified insulin receptors labeled with iodinated insulin and with ^{32}P resolved in ND-PAGE as three distinct entities. The three receptor species have the following characteristics: (i) The

low insulin receptor form shows intense labeling with iodinated insulin. It manifests basal autophosphorylation stimulated by insulin. (ii) The middle form has a similar intense labeling with iodinated ligand but does not possess the capacity to be phosphorylated. (iii) The high insulin receptor form exhibits a lower capacity to be labeled with insulin, requiring covalent binding to withstand electrophoresis; it shows pronounced insulin-stimulated autophosphorylation. Furthermore, these properties depend strongly on the temperature of incubation with the hormone. Indeed, at 22 °C the incubation length modifies neither labeling by iodinated ligand of the three forms nor autophosphorylation of the low and high species. In contrast, at 37 °C with increasing incubation length with hormone low and high molecular forms manifest decreasing labeling by radioactive insulin and declining autophosphorylation, while the middle species becomes more labeled with time.

For the different receptor species a striking uncoupling appears to exist between receptor labeling with iodinated hormone and receptor autophosphorylation properties. Labeling with ^{125}I-insulin is not modified by the phosphorylation status; differential labeling of receptor species seems therefore to depend directly on receptor aggregation (Figure 7). Further, phosphorylation of hormone–receptor complexes does not influence their aggregation (Figure 8). Comparison of the labeling patterns with ^{125}I-insulin and with ^{32}P suggests that the middle receptor species may represent a configuration in which the receptor ATP binding site and/or the autophosphorylation site(s) is (are) not accessible. Given the established role of receptor kinase in insulin action, this receptor species is unlikely to be involved in hormone signaling but could be involved in regulation of hormone binding. Further, the presence of receptor entities with different affinities could account for the curvilinearity of the Scatchard plot displayed by solubilized insulin receptors (Sweet et al., 1987a).

Concerning the nature of the three receptor species, the low molecular mass receptor form represents very likely the receptor $\alpha_2\beta_2$ monomer. The medium and high molecular mass species can be recognized as oligomers of $\alpha_2\beta_2$ with the same composition, but with distinct electrophoretic behavior; alternatively, the medium form could correspond to a dimer, $(\alpha_2\beta_2)_2$, and the high species to an oligomer, $(\alpha_2\beta_2)_n$ with $n > 2$.

The existence of a possible insulin-regulated relationship between the receptor entities was searched for. An apparent transition from low and high forms to the middle form at 37 °C could have been suspected from experiments with iodinated insulin (Figures 1–3). However, the lack of a detectable hormonal effect on ^{32}P-labeled receptors demonstrated in Figures 4 and 5 does not support an insulin-induced conversion within the studied time frame. Phosphorylation could somehow stabilize different receptor forms and prevent molecular transition. However, this possibility seems remote since phosphorylated receptor species are labeled with iodinated ligand in the same manner as nonphosphorylated receptors (Figure 7).

Our findings do not exclude that in intact cells there exist dynamic relations between the various states, which could be prevented or destroyed in cell-free systems. With fluorescent analogues of insulin, it was shown that hormone–receptor complexes are initially dispersed on the cell surface and then undergo temperature-dependent receptor aggregation, which can be induced by insulin (Schlessinger et al., 1978) or anti-receptor antibody (Schlessinger et al., 1980). The high species we detected on ND-PAGE could represent such an aggregate. Further, in intact cells, this receptor form could be produced by insulin present in serum-containing culture medium, and it could be a phosphorylation substrate for the low species receptor kinase. This highest receptor form appears as the most heavily phosphorylated species, which is compatible with the concept of a role of receptor oligomerization in kinase activation, and consequently in insulin action. Further, the observation that aggregated receptor forms are strongly phosphorylated is reminiscent of recent findings on insulin-mediated receptor downregulation. Thus, Crettaz et al. (1984) described a high molecular mass receptor state, which similarly to our high molecular mass receptor species exhibits low binding affinity and strong autophosphorylation and which is preferentially lost during insulin-induced downregulation. Conversely, kinase-defective insulin receptor mutated on the ATP binding site fails to mediate internalization and does not undergo short- or long-term downregulation in response to insulin (Russell et al., 1987).

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Orientation of the N-Terminal Region of the Membrane-Bound ADP/ATP Carrier Protein Explored by Antipeptide Antibodies and an Arginine-Specific Endoprotease. Evidence That the Accessibility of the N-Terminal Residues Depends on the Conformational State of the Carrier†

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ABSTRACT: Two peptides corresponding to the amino acid sequences 1–11 (N-terminal peptide) and 288–297 (C-terminal peptide) of beef heart ADP/ATP carrier have been synthesized. After coupling to ovalbumin, they were injected into rabbits to raise polyclonal antibodies. The specificities of the generated antibodies were tested by enzyme-linked immunosorbent assay (ELISA) and (or) Western blot. Anti-N-terminal antibodies and anti-C-terminal antibodies reacted specifically with the corresponding peptide. However, only anti-N-terminal antibodies reacted with the isolated ADP/ATP carrier; they also reacted with the membrane-bound carrier in freeze–thawed mitochondria and mitoplasts, indicating that the first 10 amino acid residues of the membrane-bound carrier in mitochondria face the cytosol. On the basis that the ADP/ATP carrier can adopt two conformations, one trapped by carboxyatractyloside (CATR conformation) and the other by bongkrekic acid (BA conformation), the reactivity of the anti-N-terminal antibodies to the ADP/ATP carrier in mitoplasts or freeze–thawed mitochondria was tested for each conformation of the carrier. Only in the CATR conformation was the N-terminal region of the membrane-bound carrier reactive to the N-terminal antibodies; the contrasting weak reactivity of the carrier in the BA conformation suggested that the transition from the CATR conformation to the BA conformation results in a restricted conformation of the peptide chain corresponding to the first 10 amino acid residues or its partial burying in the lipid bilayer. These immunological data were complemented by enzymatic data pertaining to proteolysis of the membrane-bound ADP/ATP carrier by an arginine-specific endoprotease. Enzymatic cleavage of the carrier occurred in inside-out submitochondrial particles, but not in right-side-out particles, yielding a large fragment of $M_r \simeq 25\,000$ that was immunodetected on Western blot by anticarrier antibodies but not by anti-N-terminal antibodies. These results demonstrated that the arginine-specific endoprotease had access to the matrix face of the inner mitochondrial membrane, at Arg 30 or at Arg 59. Thus, it appears that in intact mitochondria the sequence of the ADP/ATP carrier including Arg 30 or Arg 59 protrudes into the matrix space, whereas the N-terminal segment corresponding to the first 10 amino acid residues protrudes into cytosol, and the intermediate, rather hydrophobic, sequence spanning residues 9–28 transverses the lipid bilayer of the inner mitochondrial membrane.

Much interest is currently devoted to the analysis of the spatial organization of membrane-embedded proteins by chemical modification, proteolysis, and the use of site-specific immunological probes [for review, see Ovchinnikov (1987)]. These approaches are presently applied in our laboratory to the topographical study of the ADP/ATP carrier, an intrinsic protein of the inner mitochondrial membrane that catalyzes the import of cytosolic ADP into the matrix space of mito-

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