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CONFORMATIONAL STATES OF THE INSULIN RECEPTOR*

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SUMMARY: Insulin binding to the α-subunit of the purified insulin receptor changed the interaction between β-subunits. This conformational change was demonstrated after labeling the receptor's β-subunit by autophosphorylation in the absence of insulin, and then crosslinking the subunits to each other with bis(sulfosuccinimidyl)suberate. The covalent oligomers were resolved by reduction and denaturing gel electrophoresis. Insulin increased the rate of crosslinking, especially the formation of β-β dimers. These results support a conformational change following insulin binding, and may reflect the insulin-induced activation of autophosphorylation. © 1988 Academic Press, Inc.

The insulin receptor is a disulfide-linked heterotetramer. The two 135 kDa α-subunits are located on the cell surface and specifically bind insulin. Each of the two β-subunits (95 kDa) has both an extracellular domain, disulfide-bonded to the α-subunit, and an intracellular domain with protein-tyrosine kinase activity. Insulin stimulation of this activity is essential for certain cellular responses to insulin. The insulin-stimulated autophosphorylation of the receptor's β-subunit is preserved in the purified receptor (reviewed in 1,2). Therefore, with purified insulin receptor it is possible to investigate the question: Does insulin binding to the α-subunit stimulate the protein kinase activity of the β-subunit through a conformational change? To

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The abbreviations used: are BS3, bis(sulfosuccinimidyl)suberate; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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establish experimental support for a conformational change, we examined the rate of chemical crosslinking of the receptor's subunits.

MATERIALS AND METHODS

^{32}P was obtained from ICN Radiochemicals; ^{125}I-(A^{14}Tyr)-insulin was from Amersham (≈ 2000 Ci/mmol). Bis(sulfosuccinimidyl)suberate was from Pierce. [γ-^{32}P]ATP was synthesized following the method of Walseth and Johnson (3) and purified (4). Insulin derivatives were synthesized by published procedures: $N^{\alpha B1}$-(biotinyl-ϵ-aminocaproyl)insulin (5), lacking a free B1-primary amino group, and des-pentapeptide(B^{26-30})-[Tyr^{B25}-α-carboxamide]insulin (6), lacking the ϵ-amino group of LysB29. The insulin receptor was purified from 3T3-L1 cultured mouse adipocytes (7) and freed of biotinylated insulin by rechromatography on wheat germ agglutinin Sepharose. The insulin-free receptor was autophosphorylated by incubation at room temperature, pH 6.9, for 15 min, with 5 mM $Mn(CH_3CO_2)_2$ and 20 μM [γ-^{32}P]ATP (230,000 cpm/pmol). The ^{32}P-phosphoreceptor was re-isolated at 4°C by chromatography on BioGel P-6DG and sucrose density gradient centrifugation (5-20% sucrose (8)). The ^{32}P-phosphoreceptor was recovered quantitatively in two fractions. The buffer was adjusted to 50 mM HEPES , 1 mM EDTA, 0.12% Triton X-100 (w/v), pH 8.5 by dilution and subsequent concentration using a Centricon 30 (Amicon). The ^{32}P-phosphoreceptor, thus separated from free ATP and metal, was incubated or not with 1 μM insulin for 15 min at room temperature. The crosslinking was performed with 0.5 mM BS3 at room temperature, and the reaction quenched at fixed time intervals with 2-aminoethanol (9). Each sample was mixed with an equal volume of 1% sodium dodecylsulfate in 6 M urea, 0.1 M sodium phosphate and 60 mM DTT, pH 7. The samples were heated for 5 min at 60° C, and the crosslinked oligomers separated by electrophoresis in the system of Weber and Osborn (10); 4% total acrylamide (25:1, acrylamide:bis). The dried slab-gels were exposed to Kodak X-Omat AR 5 film (15 hours at -46° C without intensifying screen). Gel segments were excised according to the autoradiogram and counted in scintillant. Radioactivity in each form was calculated as a percentage of the total radioactivity in each lane.

Unphosphorylated receptor (aporeceptor) was crosslinked to ^{125}I-(A^{14}Tyr)-insulin to label exclusively the α-subunit and crosslinking was performed as above.

RESULTS AND DISCUSSION

To show a conformational change in the insulin receptor by chemically crosslinking its subunits, we considered that the rate of crosslink formation is restricted by: 1. the number of reactive groups, and 2. these groups must be at the distance fixed by the length of the crosslinking agent. The rate of crosslinking will increase if: 1. more reactive groups become available, or 2. the same number of groups reside for a longer time at the reactive distance. In a protein, these parameters for the reactive groups are controlled by the dynamic conformation of

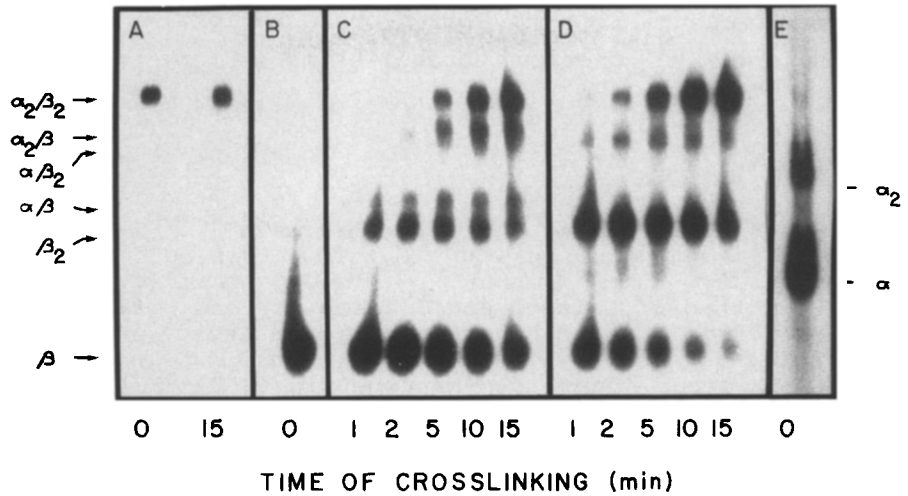

Figure 1: Cross-Linking of Insulin Receptor Subunits Following Basal Autophosphorylation. The autoradiograms show the radiolabeled oligomers and/or subunits resolved by PAGE, without (Panel A) or following reduction (Panels B-E). Panel A: the $\alpha_2\beta_2$ oligomeric state of the phosphoreceptor, without (0 min) or following crosslinking (15 min). Panel B: the migration of the ^{32}P-labeled β-subunit (apparent $M_r \approx 100$ kDa) when the quenching reagent was added prior to the BS3. Panel C: the crosslinked oligomers formed after the indicated times of crosslinking, in the absence of insulin. Panel D: the same reactions performed in the presence of 1μM insulin. Each lane (Panels A-D) contained $\approx$ 4250 $\pm$ 120 cpm of ^{32}P-labeled receptor. Panel E: the 10-min crosslinking of aporeceptor with ^{125}I-insulin.

the polypeptide backbone. Therefore, a change in the rate of subunit crosslinking would indicate a conformational change in the insulin receptor.

The homobifunctional crosslinking agent (9), BS3, was used to crosslink the insulin receptor subunits through their primary amino groups. To detect the crosslinked species resolved by PAGE, the β-subunit of the receptor was labeled with ^{32}P through its basal-state autophosphorylation reaction, and hence retains the ability to be stimulated by insulin (11). The crosslinking reactions were performed at pH 8.5 (the optimum for insulin binding (7)). This pH also favors the nonprotonated - reactive nucleophilic - state of most primary amines in proteins.

The autophosphorylated receptor occurred exclusively as the $\alpha_2\beta_2$ oligomer, and the same oligomer was detected by autoradiography after 15 minutes of crosslinking with BS3 (Fig. 1 A). Without crosslinking, but following reduction, only the β-subunit was observed (Fig. 1 B). These results were found whether or not insulin was present. The control experiments confirmed: 1. a single disulfide-bonded subunit structure, prior to reduction, 2.

the crosslinking reaction per se would not generate other oligomers or free subunits, and 3. complete reduction of the receptor occurred if no crosslinking was done. Therefore, after reduction, all molecular weight species above the β-subunit result from the crosslinking reaction.

The basal subunit crosslinking was established in the absence of insulin (Fig. 1 C). Each of the six possible molecular weight species that would contain the ^{32}P-labeled β-subunit were detected: the uncrosslinked β-subunit itself, and in ascending order of molecular weight, crosslinked β_2 and $\alpha\beta$ (dimers), $\alpha\beta_2$ and $\alpha_2\beta$ (trimers) and the $\alpha_2\beta_2$ form (tetramer). Crosslinking in presence of insulin did not yield the $\alpha\beta$-dimer or the $\alpha_2\beta$ trimer (Fig. 1 D). It is possible that bound insulin masked the amino group(s) on the α-subunit that should have been accessible to the crosslinker to produce these oligomers. Rather than a conformational change, this "steric masking" argument could explain the absence of those crosslinked oligomers.

Crosslinking ^{125}I-insulin to the receptor helped identify the oligomers described above (Fig. 1 E). The labeled α-subunit and the α_2-dimer did not coincide with any oligomers found after crosslinking when the β-subunit was labeled. The crosslinked ^{125}I-insulin-$\alpha_2\beta_2$-tetramer was only faintly visible even at longer reaction times.

Using these assigned subunit compositions, the effect of insulin on subunit crosslinking was quantified (Fig. 2). The rate

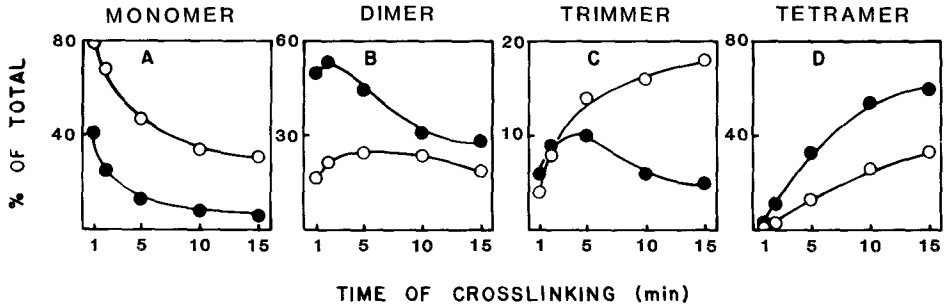

Figure 2: Time-Dependence of ^{32}P-β-Subunit Crosslinking. The reactions without insulin - open symbols; the reaction at 1 μM insulin - solid symbols. Where insulin was omitted, the "dimer" includes both $\alpha\beta$ and β_2 forms; the trimer includes both $\alpha_2\beta$ and $\alpha\beta_2$ forms. These data are from the experiment repeating that shown in Figure 1 C and D, using a different preparation of purified insulin receptor. Approximately 7000 ± 250 cpm were recovered from each lane, and the average background was 60 cpm per gel segment.

of disappearance of the β-subunit was two-fold higher in the presence than in the absence of insulin (one-min point, Fig. 2A). The expected precursor-product patterns for the dimer(s) and trimer(s) were more obvious during crosslinking in presence of insulin, consistent with a higher rate of tetramer formation (Fig. 2 D). The trimers formed without insulin present may not yet have reached the stage where conversion to the tetramer dominated, especially since the formation of crosslinked $\alpha_2\beta_2$ was slower in the absence of insulin. Finally, after a 1-min reaction period, the amount of crosslinked β_2 generated in the presence of insulin was 5-fold higher than in the absence of insulin (as a fraction of all forms, β_2 = 0.52 ± 0.03 versus 0.12 ± 0.02, plus versus minus insulin, respectively; average of three determinations). This increased crosslinking between β-subunits was the major conformational change detected.

It is possible that the crosslinking reaction might use insulin itself in forming a new covalent bridge between the subunits, since the B-chain of insulin has two free amino groups. These new reactive groups, present on the receptor when insulin is bound, might then account for the increased rate of crosslinking. To test this possibility, insulins with the B^1-Phe primary amine blocked, or lacking the B^{29}-lysine amino group (see Material and Methods), were bound to the receptor prior to the crosslinking reaction. The $\alpha\beta$-dimer and the $\alpha_2\beta$-trimer were not formed in the presence of these modified insulins. Data from the complete time courses - as in Fig. 1 C - gave results (not shown) essentially identical to data obtained with porcine insulin (± 5% of each data point in Fig. 2). Therefore insulin does not participate in a crosslinker-bridge between subunits that would be stable to reduction. This indicates that the increased rate of crosslinking is due to a change in reactive groups on the receptor's subunits.

The increased rate of subunit crosslinking is most easily explained by a conformational change in the receptor when insulin is bound. An equivalent interpretation is that the receptor exists in an equilibrium between two conformational states, and insulin favors the state more readily crosslinked. This is similar to the autophosphorylation of the receptor, which occurs in the absence of insulin, but increases when insulin is bound.

Previous reports of an insulin-dependent conformational change showed insulin-enhanced proteinase sensitivity of the

receptor's α-subunit in membranes (12) and altered sensitivity of the purified receptor to reduction in the presence of insulin (13). This sensitivity, however, was recently shown to be an effect of insulin of the disulfide linkage between the α-subunits (14). In this report, we show that the major conformational effect of insulin bound to the α-subunit is revealed by cross-linking between β-subunits (Fig. 1 D and 2 B). It may be that insulin-stimulated autophosphorylation depends upon a change in the interaction between the β-subunits. That is, more than a conformational change involving only the "αβ-half" of the receptor is essential for activation. Rather, the tetrameric receptor would be the minimum insulin-sensitive form. Indeed, insulin stimulates autophosphorylation of the $\alpha_2\beta_2$ tetrameric receptor but not the αβ-dimer formed by limited reduction (15). The subunit crosslinking method presented here should be applicable to further studies on conformational states of the insulin receptor, and other growth hormone receptors.

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