Transmembrane Signaling by the Human Insulin Receptor Kinase

RELATIONSHIP BETWEEN INTRAMOLECULAR β SUBUNIT trans- AND cis-AUTOPHOSPHORYLATION AND SUBSTRATE KINASE ACTIVATION*

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To examine the role of intramolecular β subunit trans- and cis-autophosphorylation in signal transduction, the vaccinia virus/bacteriophage T7 expression system was used to generate insulin holoreceptors composed of a kinase-defective half-receptor precursor (αβAK or αβACT) and a kinase-active half-receptor precursor (αβWT or αβWT). In the αβWT-αβACT hybrid insulin receptor, insulin stimulated a 20-fold increase in intramolecular β subunit trans-autophosphorylation, whereas cis-autophosphorylation increased only 3-fold over the basal state. Similarly, in the αβWT-αβACT hybrid insulin receptor, insulin stimulated trans-phosphorylation approximately 30-fold and cis-phosphorylation only 3-fold over the basal state. Although cis-phosphorylation of the kinase-functional αβ half-receptor was observed within these hybrid receptor species, this was not sufficient to stimulate exogenous substrate kinase activity. These data demonstrate that insulin primarily activates an intramolecular β subunit trans-autophosphorylation reaction within the insulin holoreceptor and suggest that this reaction is necessary for activation of the holoreceptor. Furthermore, our results suggest a molecular basis for the dominant-negative phenotype observed in insulin-resistant patients possessing one kinase-defective insulin receptor allele.

The insulin receptor is minimally composed of two extracellular α subunits and two transmembrane β subunits which are disulfide-linked into a functional αβ2 heterotetrameric complex (1, 2). The α subunit contains the high affinity insulin binding domain and the cytoplasmic portion of the β subunit functions as a tyrosine-specific protein kinase. In response to insulin binding, the insulin receptor β subunit undergoes autophosphorylation at several tyrosine residues. The major β subunit autophosphorylation sites have been localized to tyrosine residues 1158, 1162, 1163, 1328, and 1334 (3–5), using the nomenclature of Ebina et al. (6). Insulin-stimulated β subunit autophosphorylation occurs sequentially with Tris-phosphorylation of the 1160 region correlating with maximal substrate kinase activity (4, 7, 8). These initial events occur within a single insulin holoreceptor in an intramolecular autophosphorylation cascade (9–12); however, intermolecular cross-phosphorylation between holoreceptors has also been reported to occur under certain conditions (13–16).

Although these aspects of insulin receptor structure and function have been firmly established, several molecular details have eluded description. For example, the mechanism by which insulin binding to the extracellular α subunit(s) activates the intracellular β subunit(s) tyrosine kinase domain has not been clearly defined. Previous studies have demonstrated that specific αβ-αβ subunit interactions within the insulin holoreceptor are necessary for insulin-dependent activation of β subunit autophosphorylation and substrate kinase activity (12, 17–20). Furthermore, mutations or proteolysis which inactivate the kinase activity of only one β subunit within an αβ2 insulin holoreceptor impairs the substrate kinase activity of the functional wild type β subunit (21, 22). These data suggest that two functional β subunits are required for substrate kinase activation. Insulin binding may enable each β subunit to phosphorylate itself in an intramolecular cis reaction, or insulin may stimulate one β subunit to phosphorylate the adjacent β subunit in an intramolecular trans reaction. Whether cis-phosphorylation, trans-phosphorylation, or both are required for substrate kinase activation of the insulin holoreceptor has not yet been determined. Recently, evidence for an intramolecular cis reaction has been presented based upon the autophosphorylation of partially proteolyzed and immobilized insulin half-receptors (23). In contrast, characterization of wild type/mutant hybrid insulin receptors assembled in vitro has demonstrated an intramolecular trans-autophosphorylation pathway (22).

To address this apparent contradiction and to examine the events that occur in an intact, heterotetrameric receptor during insulin-stimulated transmembrane signaling, we have adapted the vaccinia virus/bacteriophage T7 transient expression system to generate homologous or heterologous (hybrid) insulin receptors in cultured cells (24–26). These receptor species were then used to examine the relationship between intramolecular β subunit trans- and cis-autophosphorylation and substrate kinase activation.

EXPERIMENTAL PROCEDURES

Materials—The recombinant vaccinia virus encoding T7 RNA polymerase, vTF7-3, and the vaccinia expression vector, ptM-1, were a kind gift of Dr. Bernard Moss (National Institutes of Health). HeLa and BHK-21 fibroblast cell lines were obtained from the American Type Tissue Culture Collection, and a 3T3-F442A cell line was obtained from Dr. Christin Carter-Su (University of Michigan). A plasmid containing the entire cDNA sequence of the human insulin receptor including exon 11 was constructed as described previously (27). Monoclonal antibodies directed against the human insulin receptor (85-7 and C7-1) and a monoclonal antibody directed against the
human IGF-1 receptor (αIR-3) were gifts of Dr. Kenneth Siddle (University of Cambridge) and Dr. Steven Jacobs (Burroughs Wellcome), respectively. Porcine insulin was a gift from Dr. Ronald Chance (Eli Lilly); recombinant IGF-1 was purchased from Toyobo Bio Systems and λ-HIG used for Diabetic and Endocrinology Research Center at The University of Iowa. Tran'S-label and [γ-32P]ATP were purchased from ICN and Du Pont-New England Nuclear, respectively. Methionine-free Dulbecco's minimal essential media and Lipofectin were purchased from GibCo-Bethesda Research Laboratories. The insulin receptor peptide fragment (Thr-Lys), amino acids 1134-1165, TRFYETDYYRK was a generous gift of Dr. David Coy (Tulane University, New Orleans). Poly GLU:TYR (4:1) was purchased from Sigma.

Plasmid Construction and Mutagenesis—The "Altered Sites in vitro Mutagenesis System" (Promega) was used to generate DNA encoding two previously described mutant insulin receptors; A/K (alanine for lysine substitution at amino acid 1030) and ΔCT (carboxyl-terminal 43-amino acid truncation, alanine to termination codon at position 1318) (28, 29). Briefly, an insulin receptor BamHI cDNA fragment (nucleotides 1707-4400) was cloned from pMTACE into pSelect, and single-stranded DNA was prepared. The mutagen oligonucleotides complementary to coding strand sequence, were 5'-ACGTGT-CAACGCCCAGCCCAACGCGG-3' (bases 3293-3322) for the A/K substitution and 5'-CGAGGACCTCCATCCCGGGCCCTCTATCCCTCTTGACAGTCGAGGA-3' for the ΔCT termination sequence (bases 4131-4182). Alterations in the wild type nucleotide sequence were identified. Oligonucleotide site-directed mutagenesis was performed as per manufacturer's protocol. A/K, ΔCT, and WT (wild type) human insulin receptor cDNAs were cloned into the NeoI and BamHI sites in the vaccinia expression vector, pTM-1. In these expression vectors (pTM1-WT, pTM1-A/K, pTM1-ΔCT), the NeoI restriction site is coincident with the start codon of the insulin receptor. A double mutant containing the A/K substitution and the ΔCT truncation (A/K.ΔCT) was prepared by cloning a BstXI fragment (nucleotides 894-3439) containing the A/K mutation into pTM1-ΔCT (pTM1-A/K.ΔCT).

Infection/Transfection of Cultured Cells—Healthy monolayers (approximately 10⁶ cells/100-mm dish, 2 x 10⁵ cells/150-mm dish, or 1 x 10⁵ cells/35-mm well) were infected with vTF7-3 at a multiplicity of infection of 5-10 for 1 h. After infection, cells were rinsed with serum free Ham's F-12 medium and transfected with 0.1-25 μg of CsCl-purified plasmid DNA using Lipofectin reagent (100 μg/100- or 150-mm dish, 2 x 10⁵ cells/100-mm dish) were infected with vTF7-3, transfected with receptor expression plasmids, and detergent-solubilized as described above. Insoluble material was removed by centrifugation, and the supernatant was applied to Bio-Gel A1.5m gel filtration columns (22). The peak insulin binding fractions were pooled, and receptors were further purified by immunoprecipitation with one of the insulin receptor monoclonal antibodies, 82-7 or CT-1, coupled to Affi-Gel 10 as indicated in the text.

Kinase Assays—Partially purified receptors (approximately 80 fmol of receptor/reaction) were incubated with 100 nM insulin, 50 mM HEPES, pH 7.4, 0.05% Triton X-100 for 1 h at 23 °C. Autophosphorylation was initiated by the addition of 10 μM MgCl₂, 1 mM ATP, 5 μM [γ-32P]ATP (200 μCi/nmol) (16) or by incubation with 5 mM MgCl₂, 100 μM [γ-32P]ATP (3 μCi/nmol) (22) for the indicated times. The reaction was terminated with the addition of 5 mM ATP, 5 mM EDTA, 100 mM sodium fluoride, and 10 mM sodium pyrophosphate. Receptors were then immunoprecipitated with the indicated antibody and resolved on SDS-polyacrylamide gels (7.5%) under reducing conditions. For unlabelled samples, the soluble reaction products were analyzed by SDS-PAGE under nonreducing conditions.

Results—Expression of Human Insulin Receptors—To evaluate the level of human insulin receptor expression achieved by the vaccinia virus/bacteriophage T7 system, monolayer HeLa cell cultures were first infected with recombinant vaccinia virus, vTF7-3, which encodes for the bacteriophage T7 RNA polymerase. After viral infection, the cells were transfected with various amounts of the plasmid pTM1-WT, which directs transcription of the wild type human insulin receptor from a T7 promoter (Fig. 1A). HeLa cells that were infected with virus then transfected with as little as 0.5 μg of pTM1-WT plasmid DNA demonstrated specific, cell-surface 125I-insulin binding, whereas virally infected but untransfected HeLa cells had no detectable cell surface 125I-insulin binding activity. The level of the expressed human insulin receptor increased linearly as the plasmid concentration increased and became

1 The abbreviations used are: Thr-Lys, synthetic insulin receptor peptide (amino acids 1154-1165, TRFYETDYYRK); poly GLU:TYR, synthetic 4:1 co-polymer of glutamic acid:tyrosine; A/K, alanine for lysine substitution at position 1030; ΔCT, alanine to termination codon at position 1318; pTM1-WT, double mutant containing both the A/K substitution and carboxyl-terminal truncation; WT, wild type; BHK-21, baby hamster kidney; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
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FIG. 1. Expression of wild type human insulin receptors with the vaccinia virus/bacteriophage T7 system in HeLa cells. A, cells (1 x 10^6) were infected with vTFF-7-3 and transfected with the indicated amounts of plasmid DNA encoding wild type insulin receptor (pTM1-WT). The cells were incubated for 16 h after transfection, and cell surface ^125I-insulin binding activity was determined as described under "Experimental Procedures." B, cells (1 x 10^6) were infected with vTFF-7-3 and transfected with pTM1-WT (5 μg), and cell surface ^125I-insulin binding activity was determined as indicated times following transfection.

saturated at 2 μg DNA/well. Cell surface ^125I-insulin binding activity slightly decreased as the amount of plasmid DNA increased from 5 to 25 μg. The appearance of cell-surface insulin binding activity was extremely rapid following transfection with pTM1-WT (Fig. 1B). ^125I-Insulin binding was detected as early as 2 h after transfection and increased linearly over the next 6-h period. Steady-state levels of insulin binding were observed 12-16 h after transfection and resulted in approximately 5 x 10^8 to 5 x 10^9 insulin receptors/cell (data not shown). Wild type insulin receptor produced by the vaccinia virus/bacteriophage T7 system was essentially identical to insulin receptor purified from human placenta membranes with regard to heterotetramer assembly and transport to the plasma membrane, mature sialic acid glycosylation, high affinity insulin binding, intramolecular auto-phosphorylation, and insulin-stimulated tyrosine kinase activation (data not shown).

Several groups have demonstrated that cultured cell lines expressing both insulin and IGF-1 homologous receptor complexes also express various amounts of hybrid insulin/IGF-1 receptors (32-35). Since HeLa cells contain endogenous IGF-1 receptors, we examined whether virally expressed insulin receptors could form hybrids with pre-existing IGF-1 receptors. HeLa cells were infected with vTFF-7-3, transfected with the wild type insulin receptor expression plasmid, solubilized, and immunoprecipitated with either 83-7, an insulin receptor-specific monoclonal antibody, or aIR3, an IGF-1 receptor-specific monoclonal antibody (Table I). ^125I-Insulin binding activity was not detected in control HeLa cells and therefore could not be immunoprecipitated with either monoclonal antibody. In contrast, ^125I-IGF-1 binding in control (infected only) HeLa cells was specifically immunoprecipitated by aIR3 (89%) but not by 83-7 (8%). Expression of wild type insulin receptors resulted in insulin binding activity which was specifically immunoprecipitated by 83-7 (87%) but not by aIR3 (6%). Similarly, ^125I-IGF-1 binding was only immunoprecipitated by the IGF-1 receptor monoclonal antibody (85%) and not by the insulin receptor monoclonal antibody (5%). Immuno-depletion of insulin binding activity exclusively by 83-7 and IGF-1 binding activity exclusively by aIR3 demonstrated that hybrid receptor formation between the transiently expressed insulin receptors and the endogenous IGF-1 receptors did not occur under these conditions.

Formation of Hybrid Insulin Receptors Requires Co-translation of Two Distinct Receptor Precursors—Since vaccinia virus infection inhibits host protein synthesis (25), we hypothesized that heterologous association of hybrid receptors by this transient transfection procedure would require the simultaneous translation of two distinct receptor species. We therefore transfected HeLa cells with two different insulin receptor plasmids, one encoding a kinase-defective, full-length β subunit (pTM1-A/K, β subunit M, 95,000) and another encoding a carboxyl-truncated but functional β subunit (pTM1-ΔCT, β subunit M, 90,000) (28, 29). Receptor species were identified by immunoprecipitating ^[35S]methionine-labeled proteins and separating receptor subunits on SDS-polyacrylamide gels under nonreducing conditions (Fig. 2A) or under reducing conditions (Fig. 2B). CT-1 immunoprecipitation followed by nonreducing, 3-10% SDS-polyacrylamide gel electrophoresis revealed the specific labeling of high molecular weight protein complexes (approximately M, 400,000) isolated from cells transfected with pTM1-A/K alone (Fig. 2A, lane 1) or in combination with pTM1-ΔCT (Fig. 2A, lane 3). As expected, CT-1 did immunoprecipitate the ΔCT insulin receptor due to the absence of the carboxyl-terminal epitope in the β subunit of this insulin receptor species (Fig. 2A, lane 2). The three high molecular weight bands represent previously described, partially reduced forms of the insulin receptor that result from boiling the immunoprecipitates in the presence of SDS in order to dissociate the insulin receptor from the antibody resin (36). These data demonstrate that the insulin receptor primarily exists in cells as an αβ3 heterotrimeric state with little, if any, free α or β precursors.

To determine the subunit composition of these high molecular weight receptor species, receptors were prepared in the same manner but resolved on 7.5% SDS-polyacrylamide gels under reducing conditions (Fig. 2B). Immunoprecipitation of homologous A/K insulin receptors with the 83-7 monoclonal antibody demonstrated the specific labeling of the M, 95,000 β subunit, the M, 130,000 α subunit, and the M, 190,000 precursor bands (Fig. 2B, lane 4). Similarly, immunoprecipitation of homologous ΔCT insulin receptors with 83-7 demonstrated the presence of the receptor precursor, α and β subunits (Fig. 2B, lane 5). The ΔCT receptor β subunit displayed increased mobility compared to the A/K receptor β subunit reflecting its M, 5,000 carboxyl-terminal truncation. The carboxyl-terminal-specific monoclonal antibody CT-1 also immunoprecipitated the precursor, α subunit, and β subunit of the expressed A/K insulin receptor (Fig. 2B, lane 1). Again, as expected, CT-1 did not immunoprecipitate the ΔCT insulin receptor expressed in cells transfected with the ΔCT plasmid DNA (Fig. 2B, lane 2). However, co-expression of both A/K and ΔCT insulin receptors resulted in the immunoprecipitation of the M, 90,000 ΔCT β subunit by the
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Fig. 2. Assembly of hybrid insulin receptors in vivo requires co-transfection with two insulin receptor expression plasmids. A, cells (2 x 10⁶) were infected with vTF7-3 and transfected with pTM1-A/K (6 µg, lane 1), pTM1-ΔCT (6 µg, lane 2), or simultaneously with both pTM1-A/K and pTM1-ΔCT (3 and 9 µg, respectively, lane 3). Cells were then pulse-labeled with [³⁵S]methionine, solubilized in 50 mM Tris, pH 7.4, 1 mM EDTA, 0.1% sodium dodecyl sulfate followed by immunoprecipitation with antibody CT-1. Receptors were eluted from the antibody by boiling in Laemmli sample buffer and were resolved on a 3-10% SDS-polyacrylamide gel under nonreducing conditions. This is a representative autoradiograph which was independently performed five times.

Fig. 3. Effect of various precursor expression levels on the formation of hybrid insulin receptors. Cells (1 x 10⁶) were infected and co-transfected with pTM1-WT (2.5 µg) and various amounts of pTM1-A/K-ΔCT (2.5-25 µg). Cells were pulse-labeled with [³⁵S]methionine, and solubilized extracts were subjected to immunoprecipitation with CT-1. Receptors were eluted from the antibody resin by boiling in Laemmli sample buffer containing 500 mM DTT and resolved on a 7.5% SDS-polyacrylamide gel. Autoradiographic bands were quantified by scanning laser densitometry. The percentage of the αβA/K,ACT insulin half-receptor precursor assembled into a hybrid receptor complex is shown as a function of the mole fraction of the pTM1-A/K-ΔCT insulin receptor plasmid used in the transfection.

expression of A/K,ΔCT and WT receptor subtypes, hybrid receptors accounted for approximately 50% of the total receptor population immunoprecipitated by CT-1. As the mole fraction of αβA/K,ACT increased, the percentage of hybrid receptors present in the CT-1 immunoprecipitate increased. Thus, expression of one insulin receptor precursor (approximately 3-10-fold) over another receptor precursor resulted in the near quantitative association of the less abundant species into a hybrid receptor complex. Similarly, hybrid insulin/IGF-1 receptors could be randomly assembled and were quantitatively driven with a 3-10-fold excess of one receptor species over the other (data not shown). Identical results were obtained using BHK-21, HeLa, or 3T3-F442A fibroblasts (data not shown).

Intramolecular cis- Versus trans-Autophosphorylation—To determine the relative magnitude of intramolecular cis- and trans-autophosphorylation during insulin-stimulated transmembrane signaling, αβA/K*αβΔCT hybrid insulin receptors were obtained by infection with vTF7-3 and co-transfection with equal amounts of the pTM1-A/K and pTM1-ΔCT insulin receptor expression plasmids (see Fig. 4). Hybrid αβA/K*αβΔCT heterotetramers and homologous αβA/K*αβA/K heterotetramers were then autophosphorylated in the presence or absence of insulin and selectively immunoprecipitated with the carboxy-terminal-specific CT-1 monoclonal antibody (Fig. 5). Consistent with previously reported data, the homologous A/K insulin holoreceptors were completely devoid of β subunit autophosphorylation activity both in the absence (Fig. 5A, lanes 11 and 12) and presence (Fig. 5A, lanes 13 and 14) of insulin. In contrast, β subunit autophosphorylation was observed in the αβA/K*αβΔCT hybrid receptors in a time-dependent manner. In the absence of insulin, autophosphorylation of both the M, 95,000 A/K and M, 90,000 ΔCT β subunits was essentially identical with respect to both rate and extent of phosphate incorporation (Fig. 5A, lanes 6-10), with only a small amount of M, 90,000 β subunit labeling observed. The M, 190,000 insulin receptor precursor
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Fig. 4. Schematic representation of insulin hybrid receptors. A, heterologous αβA/K-αβACT insulin hybrid receptors. B, heterologous αβW-T-αβA/K,ACT insulin hybrid receptors. The β subunit ATP binding domain is represented as an open rectangle; the A/K1030 homologous tors. The phosphorylation of the tors expressed in HeLa, BHK-21, and 3T3-F442A fibroblasts retained in both solubilized and solid-phase (immobilized immunoprecipitate) kinase assays as well as with hybrid receptors expressed in HeLa, BHK-21, and 3T3-F442A fibroblasts (data not shown).

Although these data are consistent with insulin activation of trans-autophosphorylation, the ΔCT β subunits within αβA/K-αβACT hybrid heterotetrameric complexes were determined by scanning laser densitometry (Fig. 5B). In the absence of insulin, the initial rate of both cis- and trans-autophosphorylation was similar, whereas in the presence of insulin the initial rate of trans-autophosphorylation of the M, 95,000 A/K/ΔCT β subunit was increased approximately 20-fold. In contrast, insulin stimulated the initial rate of cis-phosphorylation of the M, 90,000 ΔCT β subunit only approximately 3-fold. Essentially identical results were obtained in both solubilized and solid-phase (immobilized immunoprecipitate) kinase assays as well as with hybrid receptors expressed in HeLa, BHK-21, and 3T3-F442A fibroblasts (data not shown).

As observed for the αβA/K-αβACT hybrid insulin receptor samples, autophosphorylation of the M, 190,000 insulin receptor precursor also occurred in a time- and insulin-dependent manner (Fig. 6A). As controls, the homologous A/K/ΔCT insulin holoreceptors were virtually devoid of β subunit autophosphorylation activity in the presence of insulin at every time point examined (Fig. 6A, lanes 1–5). Quantitation of the β subunit intensity on the autoradiogram within αβW-T-αβA/K,ACT hybrid receptors by scanning laser densitometry revealed that insulin stimulated the initial rates of trans-autophosphorylation (M, 90,000) 30-fold and cis-autophosphorylation (M, 95,000) 3-fold (Fig. 6B). Identical results were obtained when the αβW-T-αβA/K,ACT hybrid insulin receptors were purified from either HeLa, 3T3–442A, or BHK-21 fibroblasts and when the kinase assays were performed with either solubilized or immobilized receptor preparations (data not shown).

Since the pattern of β subunit autophosphorylation within αβW-T-αβA/K,ACT and αβA/K-αβACT hybrid receptors was the same whether the assay was performed with solubilized or immobilized receptors, cross-phosphorylation, the phos-
in heterologous \(\alpha_1\kappa,\alpha_2\kappa,\gamma\) insulin receptors were prepared using the vaccinia virus/bacteriophage T7 expression system and partially purified by Bio-Gel A1.5m gel filtration chromatography as described under “Experimental Procedures.” Homologous \(\alpha_2\kappa,\alpha_3\kappa,\Delta\) and \(\alpha_3\kappa,\alpha_1\kappa,\Delta\) insulin receptors were subjected to autophosphorylation in the presence of 100 nM insulin. Receptors were immunoprecipitated with the 83-7 monoclonal antibody and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

Substrate Kinase Activity of Wild Type/Mutant Hybrid Insulin Receptors—In both the \(\alpha_3\kappa,\alpha_2\kappa,\Delta\) and the \(\alpha_3\kappa,\alpha_1\kappa,\Delta\) hybrid insulin receptors, the predominant insulin-stimulated event was an intramolecular trans-phosphorylation of the kinase-inactive \(\beta\) subunit by the kinase-active \(\beta\) subunit. Although cis-autophosphorylation of the kinase-active \(\beta\) subunit occurred to a lesser extent and with slower kinetics than trans-autophosphorylation, the possibility existed that this reaction could result in substrate kinase activation. We therefore examined whether this cis-autophosphorylation could activate the exogenous substrate kinase activity (poly GLU:TYR phosphorylation) of the hybrid insulin receptor species (Fig. 8). As previously reported for in vitro assembled hybrid insulin receptors (22), the in vivo assembled \(\alpha_3\kappa,\alpha_2\kappa,\Delta\) hybrid receptors were substrate kinase-inactive even in the presence of saturating insulin concentrations (Fig. 8A). Similarly, the \(\alpha_3\kappa,\alpha_1\kappa,\Delta\) hybrid insulin receptors were essentially substrate kinase-inactive (Fig. 8A). As expected, homologous \(\alpha_2\kappa,\alpha_3\kappa,\Delta\) and \(\alpha_3\kappa,\alpha_1\kappa,\Delta\) insulin receptors displayed a 3–5-fold stimulation of substrate kinase activity in the presence of insulin, whereas the homologous \(\alpha_3\kappa,\alpha_1\kappa,\Delta\) insulin receptors were completely devoid of substrate kinase activity (Fig. 8A and data not shown). Identical results were observed using the Thr-Lys synthetic peptide as a substrate for each homologous and heterologous receptor species (data not shown). To determine if the slow cis component of autophosphorylation could activate \(\alpha_2\kappa,\alpha_1\kappa,\Delta\) hybrid insulin receptors, receptors were incubated with ATP for various times in the presence or absence of insulin before the Thr-Lys peptide substrate was added to the reaction. Preincubating homologous \(\alpha_2\kappa,\alpha_3\kappa,\Delta\) and \(\alpha_3\kappa,\alpha_2\kappa,\Delta\) insulin receptors with insulin for 1 h followed by ATP for 2 min was sufficient for maximal stimulation of substrate phosphorylation (Fig. 8B). In contrast, even after a 60-min preincubation with ATP, the \(\alpha_2\kappa,\alpha_1\kappa,\Delta\) hybrid insulin receptors display a relatively low level of substrate kinase activity which was unaffected by insulin. These data demonstrate that
FIG. 8. Exogenous substrate kinase activity of the wild type and hybrid insulin receptors. A, homologous $\alpha_2\beta_2$, heterologous $\alpha_2\beta_2\gamma_2\delta_2$ and $\alpha_2\beta_2\delta_2\gamma_2$ insulin receptors were prepared using the vaccinia virus/bacteriophage T7 expression system and partially purified by Bio-Gel A 1.5 m gel filtration chromatography as described under "Experimental Procedures." These partially purified preparations were then immunoprecipitated with the carboxy-terminal-specific monoclonal antibody CT-1 and autophosphorylated in the absence (open box) or presence (shaded box) of 100 nM insulin for 6 min. The substrate kinase reactions were initiated by adding 2 mg/ml poly GLUTYR and terminated after 30 min as described under "Experimental Procedures." These results are expressed as GLUTYR phosphorylation activity (mean cpm ± S.D.) normalized for amount of receptor present (specific $^{32}$P-insulin binding, cpm) obtained from multiple experiments with the $\alpha_2\beta_2\gamma_2\delta_2$ (n = 4); $\alpha_2\beta_2\gamma_2\delta_2\gamma_2\delta_2$ (n = 2), and $\alpha_2\beta_2\delta_2\gamma_2\delta_2\gamma_2$ (n = 2). B, homologous $\alpha_2\beta_2\gamma_2\delta_2$ insulin receptors (open symbols) and hybrid $\alpha_2\beta_2\delta_2\gamma_2\delta_2\gamma_2$ insulin receptors (closed symbols) were prepared and purified as described above. The receptor species were immunoprecipitated with 83-7 and autophosphorylated in the absence (open symbols) or presence (solid symbols) of 100 nM insulin. At various times during the autophosphorylation period, substrate phosphorylation was initiated by the addition of 1 mM Thr-Lys for 30 min as described under "Experimental Procedures." This is a representative experiment of duplicate determinations independently performed two times.

the insulin-stimulated cis-autophosphorylation within an $\alpha_2\beta_2$ heterotetrameric holoreceptor does not result in substrate kinase activation.

DISCUSSION

Recently, several studies have demonstrated the presence of hybrid insulin/IGF-1 receptors in human placenta as well as various cultured cell lines (32-35). The proportion of homologous receptors compared to heterologous hybrid receptors identified in these studies appears to be variable and may reflect the relative expression of insulin and IGF-1 receptor precursors. The presence of variable amounts of homologous and heterologous insulin and IGF-1 receptors severely complicates the interpretation of studies examining insulin receptor structure-function relationships as well as studies examining the biological signaling by both insulin and IGF-1.

To exclusively generate homologous insulin receptors or defined combinations of homologous and hybrid receptors, we utilized the vaccinia virus/bacteriophage T7 expression system. Because host protein synthesis is inhibited during vaccinia virus infection, formation of hybrid receptors between endogenous host receptor precursors and transfected receptor precursors is not observed (Table I). Hybrid receptors are generated, however, by co-transfecting cells with two different insulin receptor expression plasmids (Fig. 2). Moreover, the proportion of homologous receptors and hybrid receptors expressed on the cell surface can be manipulated by altering the molar ratios of the two plasmids used in the transfection (Fig. 3). We have found that the general properties of transfected, wild type insulin receptors generated by the vaccinia virus/bacteriophage T7 expression system are identical to those of the native insulin receptors isolated from human placenta and various cell lines. This includes a normal itinerary of receptor subunit processing, assembly, and transport to the plasma membrane. Although the level of insulin receptor precursor protein is substantially greater in the vaccinia virus/bacteriophage T7 expression system than that typically observed in cells expressing normal amounts of insulin receptor, it is comparable to that observed in stable cell lines expressing high levels of insulin receptor and in transiently transfected cells expressing high levels of insulin receptor from an SR-α promoter (37). Interestingly, the M, 190,000 $\alpha_2$ precursor band was not apparent on nonreducing, SDS-polyacrylamide gels but was observed on reducing, SDS-polyacrylamide gels. These data suggest that Class I disulfide bonds form early in receptor processing, and noncovalent $\alpha_2$ dimers are transient intermediates that do not appreciably accumulate in the endoplasmic reticulum. In any event, the mature cell surface $\alpha_2\beta_2$ insulin holoreceptors produced by the vaccinia virus/bacteriophage T7 expression system display typical high affinity curvilinear ligand binding as well as insulin-stimulated intramolecular autophosphorylation and exogenous substrate kinase activity.

The vaccinia virus/bacteriophage T7 expression system was used to generate and characterize the functional properties of hybrid insulin receptors in order to address the molecular basis of insulin-stimulated transmembrane signaling with regard to the specific interactions between the $\beta$ subunit cytoplasmic domains. In previous studies, we and others have reported that isolated $\alpha_2$ insulin half-receptors and partially proteolyzed $\alpha_2\beta_2$ receptor are kinase-inactive species (12, 17-21). However, the $\alpha_2\beta_2$ half-receptors regain kinase activity upon reassociation into an $\alpha_2\beta_2$ heterotetrameric state (17, 22). In addition, in vitro assembled hybrid receptors composed of a kinase-active $\alpha_2\beta_2$ half-receptor with a kinase-inactive $\alpha_2$ half-receptor are completely devoid of substrate kinase activity but display at least partial insulin-stimulated autophosphorylation (22). Similarly, Cobb et al. (38) have observed that the kinase activity of the baculovirus-expressed $\beta$ subunit cytoplasmic domain occurs via an intermolecular association. These data suggest a requirement for interaction between $\beta$ subunits within an $\alpha_2\beta_2$ heterotetramer and suggest the possible trans-phosphorylation of one $\beta$ subunit by the adjacent $\beta$ subunit as a means of activating the holoreceptor.

In contrast, it has also been reported that the baculovirus-expressed $\beta$ subunit kinase domain remains active as a monomer (39, 40). Furthermore, partial proteolysis of the $\alpha$ subunits within an $\alpha_2\beta_2$ holoreceptor followed by isolation of the resultant half-receptors was reported to generate a monomeric, kinase-active, $\alpha$ $\beta$ half-receptor species (23). These results were interpreted as evidence for an intramolecular cis-
autophosphorylation reaction within the native αβ2 insulin holoreceptor. The reasons for these discrepant results are not apparent at the present time.

Nevertheless, the data presented in this study clearly demonstrate that insulin initially activates an intramolecular trans-autophosphorylation reaction in which the kinase-active β subunit phosphorylates the adjacent kinase-inactive β subunit within a wild type/mutant αβ2 heterotetrameric insulin holoreceptor complex. Although intramolecular cross-phosphorylation (one holoreceptor phosphorylating a second holoreceptor) has been observed under certain conditions (13-16), in the two different wild type/mutant hybrid receptor species examined in this study, autophosphorylation occurred predominantly on the kinase-inactive β subunit both in solution and in solid-phase assays. Since the solid-phase assay prevents interaction between holoreceptors, these data suggest cross-phosphorylation does not occur in this system. In addition, when kinase-active holoreceptors were mixed with truncated, kinase-inactive holoreceptors, phosphorylation of the truncated, kinase-inactive β subunit was not observed (Fig. 7). Thus, these data are consistent with previous studies that demonstrate autophosphorylation is an intramolecular reaction (9-12) and establish that the primary action of insulin is to activate intramolecular β subunit trans-autophosphorylation within a single holoreceptor.

Previous studies have identified the major β subunit autophosphorylation sites as tyrosine residues 1158, 1162, 1163, 1328, and 1334 (3-5). Tyrosine phosphorylation of the 1158, 1162, and 1163 sites were found to correlate with the activation of exogenous substrate kinase activity (4, 7, 8). In contrast, phosphorylation of the carboxyl-terminal sites (1328 and 1334) has been reported to be important in mitogenic signaling (41, 42). In both of the hybrid receptor species examined, autophosphorylation of the kinase-inactive β subunit occurred at similar rates even though the M, 90,000 A/K.ΔCT β subunit lacks the carboxyl-terminal 1316 and 1322 acceptor sites. These data indicate that the primary sites of intramolecular trans-autophosphorylation occur in the 1160 region. Furthermore, the relatively small amount of intramolecular cis-autophosphorylation observed on the kinase-active β subunit was not sufficient to activate substrate kinase activity (Fig. 8). These data suggest that the cis-autophosphorylation reaction occurs on other tyrosine residues which do not play a significant role in kinase activation.

The activation of the human insulin receptor by intramolecular trans-autophosphorylation suggests a molecular basis for the dominant-negative phenotype observed in insulin-resistant patients possessing one kinase-defective insulin receptor allele (43). If the formation of hybrid receptors in such patients occurs randomly with equal expression from both insulin receptor alleles, then as much as 75% of the total population of insulin receptors would be biologically inactive. Formation of hybrid insulin/IGF-1 receptors has been documented in vivo and in vitro and could also contribute to the defects in IGF-1 responsiveness observed in insulin-resistant patients (44).

In summary, subunit interactions between insulin receptor αβ2 half-receptors are necessary for both high-affinity insulin binding and tyrosine kinase activity. The rapid insulin-stimulated phosphorylation that occurs within intact αβ2 insulin holoreceptors predominantly results from an intramolecular trans-autophosphorylation reaction in which one β subunit phosphorylates the adjacent β subunit. These data further suggest that substrate kinase activation of the insulin holoreceptor requires sequential or simultaneous intramolecular trans-autophosphorylation reactions which results in the quantitative tyrosine phosphorylation of both β subunits.

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