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Structure and Ligand Specificity of the *Drosophila melanogaster* Insulin Receptor

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The insulin-binding and protein tyrosine kinase subunits of the *Drosophila melanogaster* insulin receptor homolog have been identified and characterized by using antipeptide antibodies elicited to the deduced amino acid sequence of the α and β subunits of the human insulin receptor. In *D. melanogaster* embryos and cell lines, the insulin receptor contains insulin-binding α subunits of 110 or 120 kilodaltons (kDa), a 95-kDa β subunit that is phosphorylated on tyrosine in response to insulin in intact cells and in vitro, and a 170-kDa protein that may be an incompletely processed receptor. All of the components are synthesized from a proreceptor, joined by disulfide bonds, and exposed on the cell surface. The β subunit is recognized by an antipeptide antibody elicited to amino acids 1142 to 1162 of the human insulin proreceptor, and the α subunit is recognized by an antipeptide antibody elicited to amino acids 702 to 723 of the human proreceptor. Of the polypeptide ligands tested, only insulin reacts with the *D. melanogaster* receptor. Insulinlike growth factors type I and II, epidermal growth factor, and the silkworm insulinlike prothoracicotropic hormone are unable to stimulate autophosphorylation. Thus despite the evolutionary divergence of vertebrates and invertebrates, the essential features of the structure and intrinsic functions of the insulin receptor have been remarkably conserved.

The subunit structure of the prototypic mammalian insulin receptor, the human placental insulin receptor, has been well characterized, and its primary amino acid sequence has been deduced from cDNA cloning (5, 27). The processed receptor is a tetramer composed of two 135-kilodalton (kDa) α subunits that bind insulin and two 95-kDa β subunits that traverse the plasma membrane and, in their cytoplasmic domain, possess protein tyrosine kinase activity (5, 27). All four subunits are glycosylated and are linked to each other by disulfide bonds (12). In an effort to find a biological system susceptible to genetic analysis of the insulin receptor, we turned to *Drosophila melanogaster*. Insects are the only nonvertebrate organisms from which an insulinlike molecule with biological activity has been fully purified (25). It seemed likely, therefore, that *D. melanogaster* would possess a homolog of the mammalian insulin receptor, the *Drosophila* insulin receptor homolog (DIRH). Previous work in our laboratory has demonstrated the existence of a *D. melanogaster* membrane-associated glycoprotein, M_r 300,000 to 400,000, that binds bovine and porcine insulin with a k_D of approximately 10 nM (17). An insulin-dependent protein tyrosine kinase activity which peaks during embryogenesis was detected, as were 100- and 120-kDa proteins that could be specifically cross-linked to ^{125}I-insulin (19). Finally a genomic sequence encoding a protein homologous to the kinase domain of the human insulin receptor, as well as the human insulinlike growth factor type I (IGF-I) receptor (28), was cloned and shown to represent a single-copy gene in the *D. melanogaster* genome (18) and to hybridize to a single chromosomal band by in situ analysis (unpublished results). The amino acid sequence (1142 to 1152) of the human proreceptor was completely conserved in the cloned *D. melanogaster* genomic fragment, predicting that an antipeptide antibody (AbP2), elicited to that peptide in the human sequence (9), would be able to immunoprecipitate the β subunit of the DIRH. In agreement with that prediction, a protein of M_r 95,000 whose phosphorylation was stimulated by insulin in vitro was immunoprecipitated by AbP2 from *D. melanogaster* embryo extracts (18).

In this report we characterize the subunit structure of the DIRH in *D. melanogaster* embryos and two *D. melanogaster* cell lines by using antibodies elicited to peptide sequences in both the α and β subunit domains of the human proreceptor. We show that the DIRH is synthesized from a higher-molecular-weight precursor and reacts with mammalian insulin but not with related insulinlike growth factors, epidermal growth factor (EGF), or the insulinlike prothoracicotropic hormone recently purified from silkworms (15). These features distinguish the DIRH from a *D. melanogaster* protein that was reported to interact with both EGF and insulin (26). We conclude that the processed DIRH is an oligomer consisting of insulin-binding subunits (α) and protein tyrosine kinase subunits (β) with homology to the human insulin receptor subunits. Unlike in the mammalian receptor, a protein of M_r 170,000 that reacts with antipeptide antibodies to the kinase domain of the human insulin receptor and is phosphorylated on tyrosine residues in response to insulin is also incorporated into a receptor oligomer that is exposed on the cell surface.

MATERIALS AND METHODS

D. melanogaster (Oregon R) flies were maintained at 25°C. Embryos were collected and dechorionated as described by Elgin and Miller (7).

Cells. *D. melanogaster* Schneider S-2 cells, obtained from Peter Ross, Rockefeller University, were grown in Schneider medium (Difco Laboratories) supplemented with 10% fetal bovine serum (Difco), 2 mM L-glutamine, and 0.2% Bacto-Peptone. *D. melanogaster* Kc cells, obtained from Macy Khoeler, Harvard University, were grown in M-3 medium containing the same supplements specified for Schneider medium. Both cell lines were grown at 23°C in

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static suspension cultures (T flasks). Cell density was maintained between 10^6 and 10^7 cells per ml.

Membrane preparation. Cultured cells were harvested by centrifugation at 23°C and washed twice in phosphate-buffered saline (PBS). After this step, all procedures were carried out at 4°C. Washed cells or dechorionated embryos were disrupted in a glass-Teflon homogenizer in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.6) containing 200 mM sucrose, 25 mM benzamidine, 4 mM EDTA, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 25 μg of leupeptin per ml, 25 μg of pepstatin A per ml, 25 μg of Trasylol per ml, 40 μg of soybean trypsin inhibitor per ml, and 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates (8%, wt/vol) were centrifuged at 1,000 × *g* for 10 min. The supernatant fluid was recovered, filtered through gauze (embryo homogenates), and centrifuged at 100,000 × *g* for 30 min. The pellet was suspended (crude membrane pellet) and used directly in some experiments (see below). For immunoblotting, autophosphorylation in vitro and cross-linking of radiolabeled insulin, a membrane glycoprotein fraction was used. It was prepared by wheat germ agglutinin-Sepharose affinity chromatography (20) as described previously (17). Protein concentrations in the membrane were determined by the method of Bradford (2).

Antipeptide antibodies. Polyclonal rabbit antibodies were elicited to peptides synthesized in accordance with the deduced amino acid sequence of the human insulin proreceptor. The procedures used to synthesize the peptides and to develop the antipeptide-antibodies have been described previously (9). Antibodies to P2 (AbP2) (amino acid residues 1142 to 1162 of the human insulin proreceptor) recognize a domain of the β subunit that is autophosphorylated in response to insulin in intact cells (24) and in vitro (10). AbP3 (amino acid residues 702 to 723) are directed to the extracellular C terminus of the α subunit. AbP4 and AbP5 recognize the N terminus and C terminus, respectively, of the cytoplasmic portion of the β subunit. AbP2, AbP4, and AbP5 have been described and characterized previously (8–10). Each antipeptide antibody (including AbP3) reacts specifically with the peptide to which it was elicited, and AbP2, AbP4, and AbP5 have been shown to immunoprecipitate the intact, processed human insulin receptor. AbP6 (amino acid residues 983 to 996) were elicited and characterized in the same fashion as for AbP2, AbP4, and AbP5.

Metabolic labeling and immunoprecipitation. Kc or S-2 cells (3×10^6 to 5×10^6) were washed twice in sterile PBS and twice in methionine-free minimal essential medium. They were then suspended in the latter medium (2×10^7 to 3×10^7 cells per ml) containing 10% dialyzed fetal bovine serum (Difco) and 2 mM L-glutamine and incubated in this medium for 15 min. L-[^{35}S]methionine (0.20 mCi/ml, 1,100 to 1,250 Ci/mmol) was then added, and the incubation was continued for 90 min. Labeled cells were collected by centrifugation and suspended in supplemented Schneider or M-3 medium (see above) containing 8 mM unlabeled methionine. After 6 h of incubation, cells were collected and washed with PBS, and crude membrane fractions were prepared as described. Pellets of membranes were sonicated for 30 s (three of four pulses) at the low setting in a Branson sonifier in 50 mM HEPES buffer (pH 7.6) containing 3.5% sodium dodecyl sulfate (SDS) and 2 mM PMSF and then heated to 100°C for 3 min. Samples were diluted 1:10 in Triton-saline buffer that contained (final concentrations) 2% Triton X-100, 250 mM NaCl, 10 μg of Trasylol per ml, 10 μg of leupeptin per ml, and 0.5 mM PMSF. The final concentrations of SDS and Triton X-100 were 0.35 and 2%, respectively. Diluted samples were first cleared by simultaneous addition of nonimmune serum and protein A-Sepharose for 3 h at 4°C. The Sepharose beads were removed by centrifugation in a Microfuge (Beckman Instruments), and the supernatant fluids were recovered and incubated with immune sera (antipeptide antibodies) in the presence or absence of the corresponding peptide, for 10 to 12 h at 4°C. The immune complexes were then precipitated with protein A-Sepharose, washed as described previously (1), boiled in sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) (14). Unless otherwise indicated, immunoprecipitation with anti-phosphotyrosine antisera, elicited as described previously (6), and antisera to the purified placental insulin receptor were performed as described for the antipeptide antibodies. Gels were stained, destained, treated with En^{3}Hance (New England Nuclear Corp.), dried, and exposed to XAR film (Eastman Kodak Co.) at −70°C. The procedure for labeling cells in the pulse-chase experiments is identical to that described above. Pulse and chase times are detailed in the figure legends.

Receptor phosphorylation in vitro. Reaction mixtures of 50 μl contained 50 mM HEPES buffer (pH 7.6), 12.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.5 mM dithiothreitol, and 2 to 3 μg of wheat germ agglutinin eluate protein (see above) prepared from *D. melanogaster* embryos or *D. melanogaster* cell lines. The mixtures were incubated in the presence or absence of insulin (100 nM, unless otherwise stated) for 90 min at 4°C and then incubated for 20 min at 24°C with 40 μM [γ-^{32}P]ATP (80 cpm/fmol) added. Reactions were terminated by adding unlabeled ATP and SDS to 8 mM and 0.3% (final concentrations), respectively, and heating the mixture to 100°C for 3 min. Samples were diluted, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis as described above. Fixed and stained gels were treated with 1 N NaOH for 90 min at 55°C, dried, and exposed to XAR film at −70°C with an intensifying screen.

Cross-linking of insulin. Wheat germ agglutinin eluate (5 to 6 μg of protein) was covalently linked to ^{125}I-insulin by using the bifunctional reagent disuccinimidyl suberate (21) as described previously (4). After being cross-linked, the samples were denatured in SDS, diluted, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as outlined for the metabolically labeled *D. melanogaster* proteins.

Cell surface iodination. Kc and S-2 cells were washed three times in PBS and iodinated as described previously (13). Briefly, cells (approximately 6×10^8) were suspended in 25 ml of PBS supplemented with 20 mM glucose, lactoperoxidase (30 μg/ml), glucose oxidase (50 U/ml), and Na^{125}I (0.1 mCi/ml) and incubated at 23°C for 30 min with gentle agitation. After iodination, cells were washed three times with PBS, twice with PBS containing 0.5% bovine serum albumin, and finally once with cold PBS. The cells were then lysed in 2.5 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 5 mM EGTA, 3% SDS, and 2 mM PMSF. Lysates were clarified by centrifugation at 150,000 × *g* for 3 h. Supernatant fluids were diluted and analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography as detailed above.

Phosphoamino acid analysis. Wheat germ agglutinin eluates from Kc or S-2 cells were phosphorylated in vitro in the presence or absence of insulin and immunoprecipitated with AbP2 as described above. Washed immunoprecipitated proteins were eluted from the immunoadsorbent with 0.5 M

acetic acid, dried, hydrolyzed in 6 N HCl for 90 min at 100°C, and analyzed by electrophoresis on cellulose thin-layer plates (11).

Receptor phosphorylation in intact cells. Kc or S-2 cells (6×10^6 to 7×10^6 cells) were incubated with carrier-free $^{32}p_i$ (0.5 mCi/ml) for 4 to 5 h in phosphate-free minimal essential medium supplemented with 0.4% dialyzed bovine serum albumin and 2 mM glutamine. Labeled cells were then treated with insulin (see figure legends) for 20 min, and total cell lysates prepared in SDS were immunoprecipitated as described above for cell surface-iodinated cells. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Materials. Benzamidine, PMSF, DL-phosphotyrosine, L-phosphoserine, L-phosphothreonine, and protein A-Sepharose were purchased from Sigma Chemical Co. Dithiothreitol was obtained from Calbiochem-Behring, and disuccinimidyl suberate was from Pierce Chemical Co. HEPES, leupeptin, soybean trypsin inhibitor, and Trasylol were purchased from Boehringer Mannheim Biochemicals. [γ-^{32}P]ATP (3,000 Ci/mmol), [^{35}S]methionine (>1,000 Ci/mmol), and Triton X-100 were obtained from New England Nuclear Corp. Na^{125}I (529 mCi/mg) and carrier-free $^{32}P_i$ were purchased from Amersham Corp. Agarose-bound wheat germ agglutinin was obtained from Vector Laboratories. EGF and IGF-II were purchased from Collaborative Research. IGF-I was from AMGen, and porcine insulin was a gift from Eli Lilly & Co. Insulin was iodinated by the chloramine T method to a specific activity of 2,000 cpm/fmol

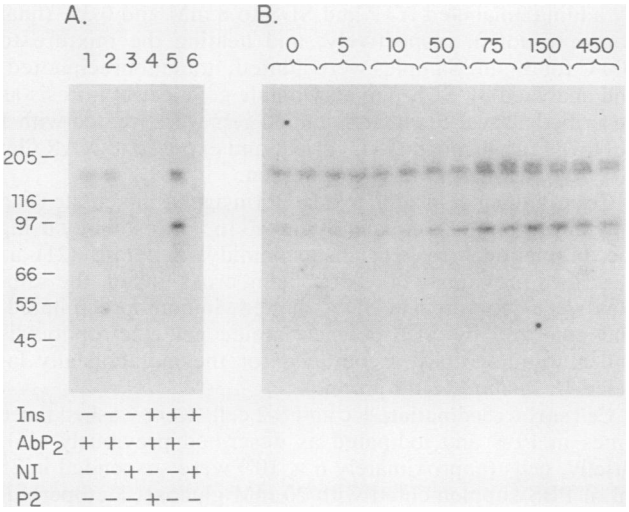

FIG. 1. Insulin-stimulated phosphorylation of *D. melanogaster* embryo proteins in vitro. (A) Wheat germ agglutinin eluate (1.5 μg of protein) derived from 0- to 15-h pooled embryos was phosphorylated in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of insulin (100 nM), immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (see Materials and Methods). Lanes 1, 2, 4, and 5 were immunoprecipitated with AbP2. Lanes 3 and 6 were incubated with nonimmune serum. In lane 4, P2 (40 μg/ml) was included during the immunoprecipitation. (B) The same fraction (3-μg portions) was phosphorylated in the presence of increasing concentrations of insulin (numbers correspond to the nanomolar concentrations of insulin). Samples were processed by immunoprecipitation with AbP2 and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The molecular mass markers (in kilodaltons) are indicated to the left of panel A. The autoradiograms were developed for 3 days at −70°C.

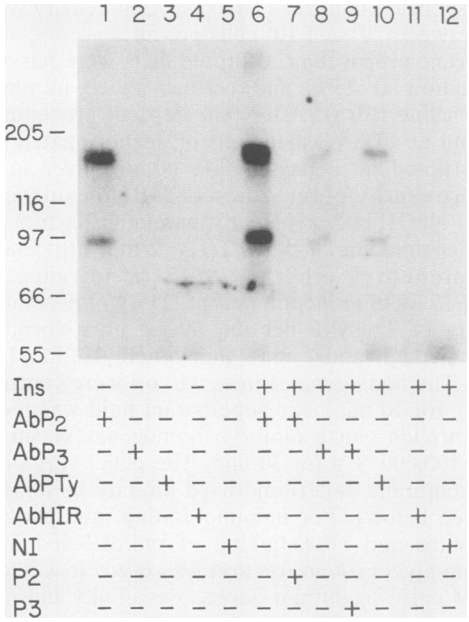

FIG. 2. Immunoprecipitation of phosphorylated *D. melanogaster* embryo proteins by anti-human insulin receptor and antiphosphotyrosine antibodies. Portions of wheat germ agglutinin eluate derived from 0- to 16-h pooled embryos (2.5 μg of protein) were phosphorylated in the absence (lanes 1 to 5) or presence (lanes 6 to 12) of 100 nM insulin, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (see Materials and Methods). Lanes 1, 6, and 7 were immunoprecipitated with AbP2; lanes 2, 8, and 9 were immunoprecipitated with AbP3; lanes 3 and 10 were immunoprecipitated with antiphosphotyrosine antibodies; lanes 4 and 11 were immunoprecipitated with a polyclonal antibody prepared against the purified human placental insulin receptor; and lanes 5 and 12 were immunoprecipitated with nonimmune rabbit serum. In lanes 7 and 9 the peptides to which the antipeptides were elicited were added during the immunoprecipitation at a final concentration of 40 μg/ml. The molecular mass markers (in kilodaltons) are indicated to the right. The autoradiogram was developed for 5 days at −70°C.

(22). Pure silkworm prothoracicotropic hormone (4K-PPTH-II) was a gift of Akinori Suzuki, University of Tokyo.

RESULTS

Cross-reactivity of antipeptide antibodies to the human insulin receptor with *D. melanogaster* membrane proteins. To further establish and characterize the DIRH, we used antipeptide antibodies elicited to sequences deduced from the human insulin proreceptor cDNA to identify cross-reactive polypeptides in *D. melanogaster* embryos and cultured cells. We initially tested a panel of seven antipeptide antibodies to the human insulin proreceptor with wheat germ agglutinin-purified membrane proteins from *D. melanogaster* embryos by using Western blot (immunoblot) analysis following disulfide reduction and SDS-polyacrylamide gel electrophoresis. Antibody P2, which recognizes amino acids 1142 to 1152 of the kinase domain (see Materials and Methods) was the only one that showed specific reactivity with a 95-kDa protein. To determine whether this 95-kDa protein was indeed homologous to the β subunit of the mammalian insulin receptor, we assayed a specific property of the insulin receptor β subunit, autophosphorylation on tyrosine residues in response to insulin (20). The results are

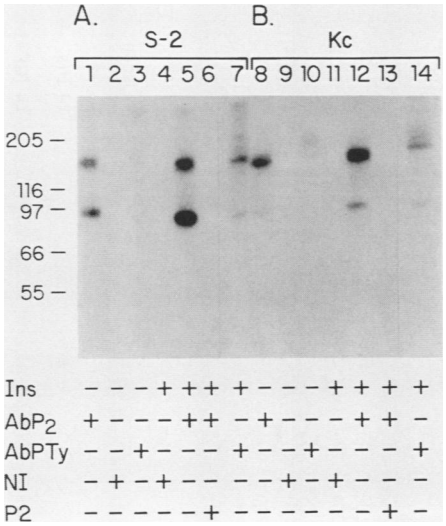

FIG. 3. Immunoprecipitation of phosphorylated proteins from cultured *D. melanogaster* cells. Portions of wheat germ agglutinin eluates (2 μg of protein) obtained from S-2 cells (lanes 1 to 7) and Kc cells (lanes 8 to 14) were phosphorylated in vitro in the absence (lanes 1 to 3 and 8 to 10) or presence (lanes 4 to 7 and 11 to 14) of 100 nM insulin and immunoprecipitated with either AbP2 (lanes 1, 5, 6, 8, 12, and 13), antiphosphotyrosine antibody (lanes 3, 7, 10, and 14), or nonimmune serum (lanes 2, 4, 9, and 11) and analyzed by SDS-polyacrylamide gel electrophoresis. P2 (40 μg/ml) was included during the immunoprecipitation in lanes 6 and 13. The molecular mass markers (in kilodaltons) are indicated to the left. The gel was exposed for 3 days at −70°C.

shown in Fig. 1 and 2. When wheat germ agglutinin eluates derived from solubilized membranes of *D. melanogaster* embryos were incubated with insulin and [γ-^{32}P]ATP and then immunoprecipitated with AbP2, the ^{32}P incorporated into the *D. melanogaster* 95-kDa protein was increased about fourfold. Surprisingly, another immunoreactive protein of 170 kDa was also phosphorylated in response to insulin (Fig. 1A). The ^{32}P associated with both proteins was resistant to alkali (see Materials and Methods), suggesting that it was incorporated into tyrosine residues (3). The specificity of the immunoprecipitation reaction is illustrated by the fact that it is quantitatively inhibited by the addition of peptide 2 (compare Fig. 1A, lanes 4 and 5).

Phosphorylation of both the 95- and 170-kDa proteins was dependent upon the concentration of insulin added (Fig. 1B). The minimal concentration of insulin required to detect an increase in the phosphorylation of both proteins was approximately 50 nM.

We next determined whether any of the other antisera directed against specific regions of the human insulin receptor could immunoprecipitate the *D. melanogaster* phosphoproteins. AbP3, a previously undescribed antipeptide antibody directed against the C-terminal portion of the α subunit (amino acids 702 to 723 of the human proreceptor), was able to immunoprecipitate the same phosphoproteins detected with AbP2 (Fig. 2). Furthermore, rabbit antiphosphotyrosine antiserum was also able to immunoprecipitate both phosphoproteins, providing additional evidence that the insulin-stimulated phosphorylation occurs on tyrosine residues.

Production of DIRH in established *D. melanogaster* cell lines. When wheat germ agglutinin eluates from three established *D. melanogaster* cell lines, Kc (Fig. 3), S-2 (Fig. 3), and S-3 (data not shown), were assayed for insulin-stimulated phosphorylation, two proteins with the same apparent molecular mass (95 kDa and 170 kDa) as those found in the embryos were insulin responsive. As in the embryos, these phosphoproteins were specifically immunoprecipitated by AbP2 and antiphosphotyrosine antibodies (Fig. 3) and AbP3 (see Fig. 8C and D). Phosphoaminoacid analysis of AbP2 immunoprecipitates derived from Kc cells (Fig. 4) and S-2 cells (data not shown) indicated that tyrosine was the only amino acid whose phosphorylation was stimulated by insulin in vitro. In the absence of insulin, the small amount of ^{32}P incorporated was also exclusively on tyrosyl residues.

Immunoprecipitation of *D. melanogaster* insulin-binding proteins by AbP2. To determine whether the insulin-binding domain of the DIRH (α subunit [17, 19]) and the 95-kDa insulin-dependent phosphoprotein were covalently linked to each other, we labeled the binding domain by cross-linking with ^{125}I-insulin and immunoprecipitated the complex with AbP2, which recognizes a sequence in the kinase domain of the human insulin receptor β subunit. When wheat germ agglutinin eluates derived from *D. melanogaster* embryos or human placenta were cross-linked to ^{125}I-insulin and immunoprecipitated with AbP2, a single radioactive band was detected (Fig. 5A). As expected, the human placenta protein was approximately 135 kDa. The principal *D. melanogaster* insulin-binding subunit had a lower molecular mass, approximately 110 kDa (lane 5). Cross-linking to ^{125}I-insulin was prevented by the addition of a 100-fold molar excess of unlabeled insulin (lanes 1 and 4). The specificity of the immunoprecipitation reaction was confirmed by the addition of excess P2 (lanes 3 and 6). A similar experiment was

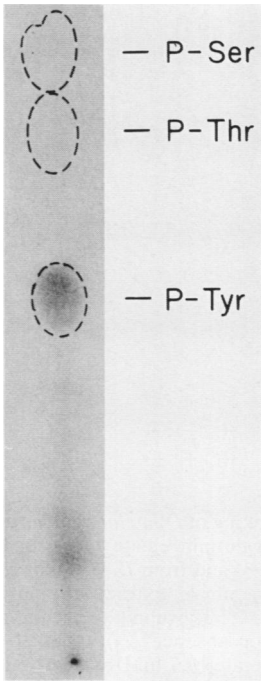

FIG. 4. Phosphoamino acid analysis of the *D. melanogaster* proteins immunoprecipitated by AbP2. A portion of wheat germ agglutinin eluate (4 μg of protein) obtained from Kc cells was phosphorylated in vitro in the presence of 100 nM insulin and immunoprecipitated with AbP2. Phosphoproteins were eluted and analyzed for phosphoamino acid content, as described in Materials and Methods.

performed with extracts derived from *D. melanogaster* cell lines (Fig. 5B). Again, a single polypeptide was detected in both S-2 (lanes 1 to 3) and Kc (lanes 4 to 6) cells. The insulin-binding subunit in S-2 cells has molecular mass of approximately 110 kDa, similar to that found in embryos. In Kc cells the binding protein has a slightly higher molecular mass, approximately 120 kDa. There was no detectable ^{125}I-insulin cross-linked to the 170-kDa protein in either embryos or cell lines. As in the case of the embryos, cross-linking of ^{125}I-insulin was prevented by the presence of an excess of unlabeled insulin and immunoprecipitation of the complex by AbP2 was abrogated by the addition of peptide P2.

Ligand specificity of the DIRH. Two approaches were used to analyze the specificity of the DIRH for insulin: the ability of putative ligands to stimulate the kinase activity associated with the DIRH (Fig. 6) and the capacity of these ligands to compete with ^{125}I-insulin for binding to the α subunit (Fig. 7). When wheat germ agglutinin eluates prepared from Kc cells (Fig. 6B) or S-2 cells (Fig. 6A) were incubated with 100 nM insulin, phosphorylation of the 95- and 170-kDa proteins was stimulated, as noted above. The same concentration of IGF-I, IGF-II, or EGF elicited no detectable change in the phosphorylation state of either protein. Even higher concentrations (750 nM) were ineffective in stimulating ^{32}P incorporation into the 95- and 170-kDa proteins (data not shown).

To further address the specificity of the DIRH, we assayed the ability of the purified insulinlike hormone 4K-PTTH-II (15), isolated from the silkworm *Bombyx mori*, to stimulate

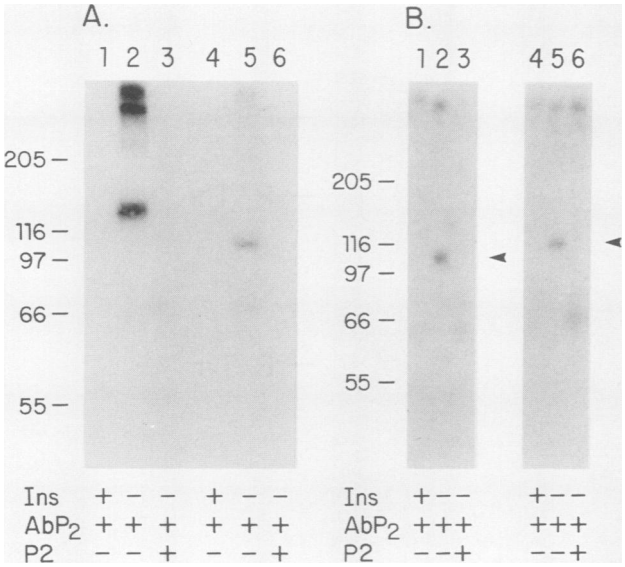

FIG. 5. Cross-linking of *D. melanogaster* proteins to ^{125}I-insulin. (A) Wheat germ agglutinin eluate (3 μg of protein) from human placenta (lanes 1 to 3) and from *D. melanogaster* embryos (5 μg of 0- to 20-h pooled embryo extract protein; lanes 4 to 6) were incubated with 10 nM ^{125}I-insulin (2,000 cpm/fmol) for 2 h at 4°C, in the presence (+) or absence (–) of unlabeled insulin (1 μM). Disuccinimidyl suberate (0.3 mM) was added for 15 min. Samples were then immunoprecipitated with AbP2, in the absence (lanes 1, 2, 4, and 5) or presence (lanes 3 and 6) of peptide 2 (40 μg/ml) and analyzed by SDS-polyacrylamide gel electrophoresis. (B) Portions of wheat germ agglutinin eluates (5 μg of protein), prepared from S-2 cells (lanes 1 to 3) and Kc cells (lanes 4 to 6) were processed as described for panel A. The molecular mass markers (in kilodaltons) are indicated to the left of each panel. Autoradiograms were exposed for 4 days at –70°C.

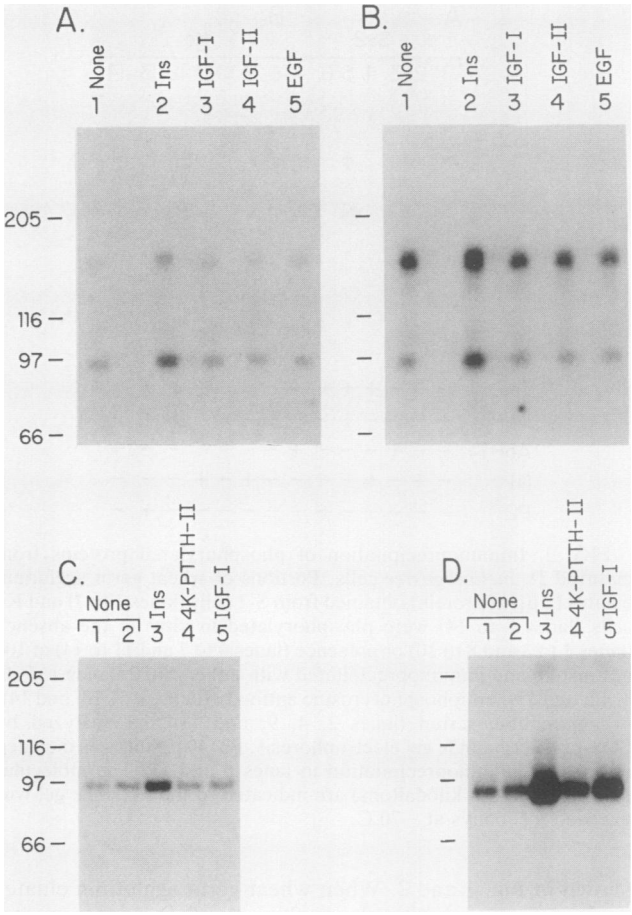

FIG. 6. Specificity of ligand-stimulated phosphorylation of the *D. melanogaster* 95- and 170-kDa proteins. Wheat germ agglutinin eluates (2 μg of protein) obtained from S-2 (A) or Kc (B) cells were phosphorylated in the absence of ligand (lane 1) or in the presence of 150 nM insulin (lane 2), IGF-I (lane 3), IGF-II (lane 4), or EGF (lane 5). Wheat agglutinin eluates from S-2 (C) or human placenta (D) cells were phosphorylated in the absence of ligand (lanes 1 and 2) or in the presence of 100 nM insulin (lane 3), 4K-PTTH-II (lane 4), or IGF-I (lane 5). All of the samples were immunoprecipitated with AbP2 and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (2 days of exposure at –70°C) as described in Materials and Methods. The molecular mass markers (in kilodaltons) are indicated to the left of panels A and C.

phosphorylation of the DIRH polypeptides as well as the β subunit of the human placental insulin receptor. While insulin stimulated the phosphorylation of both the 95- and 170-kDa proteins of S-2 cells (Fig. 6C) or Kc cells (data not shown) and the β subunit of the human insulin receptor (Fig. 6D), 4K-PTTH-II (100 nM) had no effect on either of the *D. melanogaster* proteins. It was, however, able to stimulate phosphorylation of the β subunit of the human receptor about 50%. IGF-I (100 nM) stimulated the phosphorylation of the human β subunit. This may reflect stimulation of insulin receptor autophosphorylation by the relatively high concentration of IGF-I or immunoprecipitation of both the human insulin receptor and the human IGF-I receptor by AbP2. The amino acid sequence of P2 is present in both human receptors (27, 28). In either case, the existence of an IGF-I receptorlike molecule in *D. melanogaster* seems improbable.

The results of the second method used to determine the

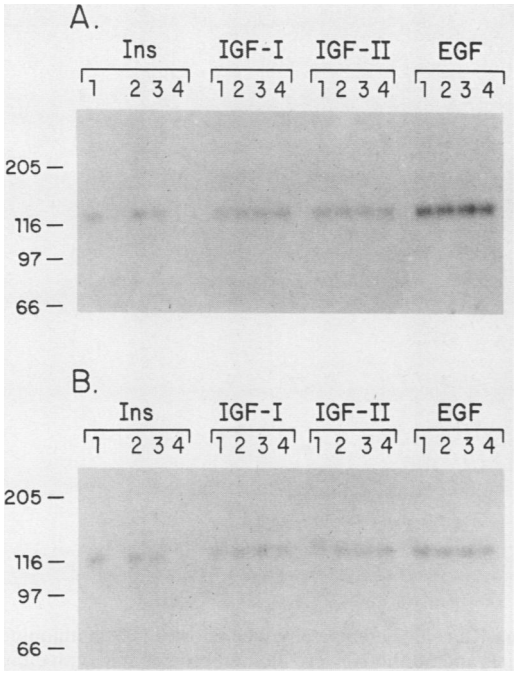

FIG. 7. Specificity of ligand binding to the DIRH. Samples of wheat germ agglutinin eluate (5 μg of protein) obtained from either Kc cells (A) or S-2 cells (B) were incubated with 12 nM ^{125}I-insulin (1,500 cpm/fmol) for 2 h at 4°C in the presence of different concentrations of unlabeled insulin, IGF-I, IGF-II, or EGF. Lanes 1, 2, 3, and 4 contain 0, 60, 120 and 1,200 nM corresponding unlabeled ligand, respectively. All the samples were immunoprecipitated with AbP2 and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as indicated in Materials and Methods. Gels were exposed for 4 days at −70°C. The molecular mass markers (in kilodaltons) are indicated to the left.

specificity of the DIRH are illustrated in Fig. 7. Extracts from Kc cells (Fig. 7A) or S-2 cells (Fig. 7B) were incubated with 12 nM ^{125}I-insulin alone (lanes 1) or with ^{125}I-insulin plus 5-fold (lanes 2), 10-fold (lanes 3), or 100-fold (lanes 4) molar excess of unlabeled ligands (insulin, IGF-I, IGF-II, or EGF). Only insulin was able to compete with the radiolabeled ligand in a dose-dependent manner. A molar excess of unlabeled over labelled insulin in a ratio of 10:1 led to a decrease in ^{125}I-insulin cross-linked to the α subunit, and a molar excess ratio of 100-fold obliterated it. No competition was observed with the other ligands at the same concentrations, confirming the specificity observed with the kinase assay. The larger amount of ^{125}I-insulin bound to the α subunit in the experiment in which EGF was used as competitor resulted from performing the experiment at a pH that was 0.2 to 0.3 pH units higher than the pH used in the insulin and IGF experiments (pH 7.2).

Subunit structure of the DIRH. To characterize the subunit structure of the DIRH, Kc cells were biosynthetically labeled with [^{35}S]methionine. Cell lysates prepared in the presence or absence of disulfide reduction (see Materials and Methods) were then immunoprecipitated with either AbP2 or AbP3. The results are presented in Fig. 8A and B. In Fig 8B, denatured, unreduced samples were immunoprecipitated with AbP2 and then subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions (lanes 3 and 4). Five polypeptides were specifically recognized by this antiserum; three of them (indicated by the arrows in Fig. 8A and B) were previously visualized by cross-linking with iodinated insulin (120 or 110 kDa) or by insulin-stimulated phosphorylation (95 and 170 kDa). The remaining two (210 and 230 kDa), indicated by the black dots, were seen only transiently upon metabolic labeling. Both of these proteins behaved in pulse-chase experiments (see Fig. 11) like receptor precursors. The protein band of similar molecular mass that was detected by AbP3 is nonspecific, since it is also immunoprecipitated by AbP3 in the presence of P3 (Fig. 8A, lanes 3 and 4; Fig. 8B, lanes 1 and 2). When samples were reduced before being immunoprecipitated with the same antiserum (Fig. 8A, lanes 1 and 2), the two precursorlike polypeptides, the 170-kDa protein and the β subunit (95 kDa), were immunoprecipitated as before, but the α subunit (110 or 120 kDa) was not. This is consistent with the predicted specificity of the antibody for the kinase domain of the β subunit. AbP2 would be expected to immunoprecipitate the dissociated β subunit and any larger polypeptide that contained an accessible P2 sequence. It would not recognize the α subunit unless it was cross-linked to a component containing the P2 sequence.

When reduced samples were immunoprecipitated with AbP3 (Fig. 8A, lanes 3 and 4), a single polypeptide of 110 kDa was detected. This protein has the same molecular mass as the protein that can be cross-linked by ^{125}I-insulin and indirectly immunoprecipitated by AbP2. The results with AbP3 are again consistent with the expected specificity for an antibody directed against the α subunit. When AbP3 was used to immunoprecipitate unreduced samples, the 170-kDa protein, the α subunit, and the β subunit were detected, predicting disulfide interactions between all three polypeptides. Interestingly, the precursorlike proteins were not immunoprecipitated by AbP3 in either reduced or unreduced samples. This suggests that AbP3 has restricted recognition for the P3 site, interacting only with the α subunit in processed species of the DIRH. The fact that AbP3 does not immunoprecipitate the 170-kDa protein in reduced samples does not therefore imply an absence of the P3 sequence in this polypeptide. Finally, an additional antipeptide antibody elicited to the ATP-binding domain of the human receptor was able to specifically immunoprecipitate both β subunit and the 170-kDa proteins of the reduced [^{35}S]methionine-labeled receptor in dissociated embryo cells (not shown).

To confirm the results shown in Fig. 8A and B, we phosphorylated portions of wheat germ agglutinin eluates from S-2 cells (Fig. 8C) and Kc cells (Fig. 8D) in the presence of insulin and immunoprecipitated them under nonreducing conditions with different dilutions of AbP3. The β subunit and the 170-kDa protein were immunoprecipitated in a concentration-dependent manner, again suggesting a covalent linkage between the two phosphoproteins and the α subunit. Supernatants from the AbP3 immunoprecipitates were further treated with AbP2. The results showed that all of the AbP2 immunoreactive β subunit and 170-kDa protein had been removed by AbP3 (data not shown). Since AbP2 recognizes both proteins independently of their state of disulfide reduction, this experiment argues that the 170-kDa protein exists principally as a component of the DIRH oligomer. Further support for this suggestion came from an analysis of the behavior of the autophosphorylated DIRH on 5% polyacrylamide gel electrohporesis (Fig. 9). Under nonreducing conditions, all of the DIRH migrated like the unreduced human insulin receptor at an M_r greater than 300,000.

Subcellular localization and biosynthesis of the DIRH. To determine whether the 170-kDa protein is a component of

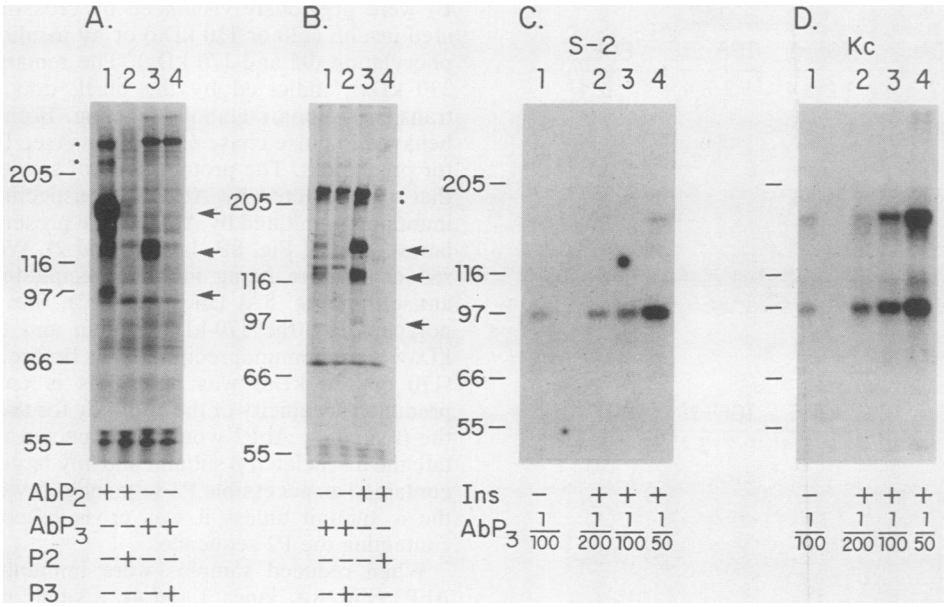

FIG. 8. Specificity of antipeptide antibodies. (A and B) Total cell lysates from Kc cells metabolically labeled with [^{35}S]methionine (0.8×10^6 to 1×10^6 cells per lane) prepared as described for iodinated cells (see Materials and Methods) were immunoprecipitated with either AbP2 or AbP3. Lysates were prepared in the presence (panel A) or absence (panel B) of 30 mM 2-mercaptoethanol (3 mM [final concentration] in the immunoprecipitation reaction). When indicated, peptides 2 and 3 (40 μg/ml) were included during the immunoprecipitation. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described in Materials and Methods. Gels were exposed for 3 days at −70°C. Subunits of the DIRH are indicated by the arrows to the right; molecular mass markers (in kilodaltons) are indicated to the left. (C and D) Wheat germ agglutinin eluate (2 μg of protein) obtained from S-2 cells (panel C) or Kc cells (panel D), was phosphorylated in vitro in the absence (lane 1) or presence (lanes 2 to 4) of 100 nM insulin and immunoprecipitated without reduction with different dilutions of AbP3. Dilutions used were 1/200 (lane 2), 1/100 (lanes 1 and 3), and 1/50 (lanes 4). Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The molecular mass markers are indicated (in kilodaltons) to the left of panel C. The gel was exposed for 2 days at −70°C. The black dots indicate the positions of the receptor precursors.

the DIRH that is exposed on the cell surface, intact cells were externally labeled with Na^{125}I and lactoperoxidase, solubilized, and then immunoprecipitated with AbP2. In both S-2 and Kc cells, three immunoreactive components were iodinated (Fig. 10). The major band is the α subunit, which is known to be an external membrane protein in mammalian cells (5, 27). The radioactivity incorporated into the 170-kDa protein was substantial in the Kc cells, but considerably less in the S-2 cells. This disparity is consistent with the relative abundance of the 170-kDa proteins in the two cell lines as judged by biosynthetic labeling (compare Fig. 8A and Fig. 11) and autophosphorylation. The lower degree of labeling of the β subunit in both cell lines is consistent with the proposed topology of the mammalian β subunit (5, 27). The latter is a transmembrane protein with only a portion of its sequence exposed to the outside of the cell.

Another method to analyze the nature of the 170-kDa protein is by biosynthetic labeling with [^{35}S]methionine. When S-2 cells were labeled for 60 min, two insulin proreceptorlike molecules, indicated by asterisks, were detected with AbP2 (Fig. 11). The labeling in both proteins was diminished by the addition of unlabeled methionine, with the concomitant appearance of the three labeled components of the mature receptor. These results strongly suggest that, like its mammalian counterpart, the DIRH is formed from a higher-molecular-weight precursor (4, 12). The disposition of α and β subunits of the DIRH in the plasma membrane is consistent with that found in mammalian cells (5, 27). On the other hand, the presence of the 170-kDa protein in *D. melanogaster* embryos and cultured cells and the observations that this protein is associated with processed α and β subunits, exposed on the cell surface, and synthesized with the same kinetics as the α and β subunits, suggest that it is a component of the DIRH. We have not, however, established whether the 170-kDa protein is a unique polypeptide that contains a P2 site and a P6 site like the β subunit of the DIRH or whether it is transcribed from the same DIRH gene and is differentially processed at the mRNA or protein levels. Since the DHIR appears to be a single gene in *D. melanogaster* (18), the latter is the more likely possibility. None of the components of the DIRH, including the 170-kDa protein, are immunoprecipitable by antibody to the human EGF receptor.

Phosphorylation of the DIRH in response to insulin in intact cells. Figure 12 shows the effect of insulin on the phosphorylation of the DIRH in intact Kc (Fig. 12A) and S-2 cells (Fig. 12B). In this experiment, ^{32}P-labeled cells were incubated for 20 min with insulin. The DIRH in both cell lines was phosphorylated in response to insulin. In S-2 cells, insulin stimulated the phosphorylation of both the β subunit and the 170-kDa protein at the lowest concentration of hormone tested (2 nM). Phosphorylation of both proteins was maximal at 10 nM insulin. In Kc cells, higher concentrations of insulin were required. It should be noted, however, that basal phosphorylation (in the absence of added insulin) was much higher in Kc than in S-2 cells. Although we do not know the biological consequences of the phosphorylation of the DIRH, the experiments with intact cells suggest that it may occur in the intact organism.

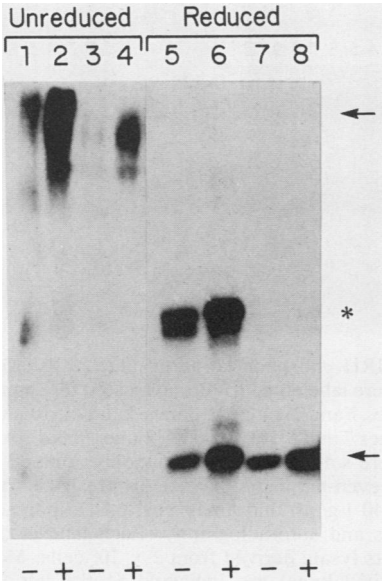

FIG. 9. Electrophoretic analysis of the DIRH under nonreducing conditions. Wheat germ agglutinin eluate (5 μg of protein) from Kc (lanes 1 and 2) or S-2 (lanes 3 and 4) cells was phosphorylated in vitro in the presence (+) (lanes 2 and 4) or absence (−) (lanes 1 and 3) of 100 nM insulin and immunoprecipitated with AbP2. Immunocomplexes were dissociated in Laemmli sample buffer that contained either 100 mM dithiothreitol (reduced samples) or no reducing agent (unreduced samples) and were analyzed by SDS-polyacrylamide gel electrophoresis in a 5% gel. The autoradiogram was developed for 2 days at −70°C. The arrows to the right indicate the positions at which the β subunit (lower arrow) and the unreduced form (upper arrow) of the human insulin receptor migrate. The asterisk indicates the migration position of the 170-kDa subunit of the DIRH.

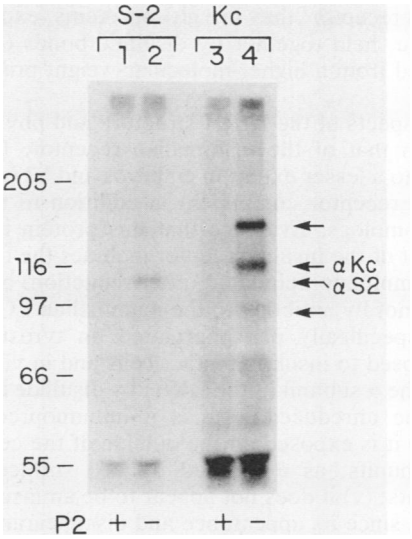

FIG. 10. Immunoprecipitation of cell surface radioiodinated polypeptides by AbP2. Total cell lysates prepared from iodinated S-2 cells (lanes 1 and 2) or Kc cells (lanes 3 and 4) (see Materials and Methods) were immunoprecipitated with AbP2 in the absence (lanes 2 and 4) or presence (lanes 1 and 3) or 40 μg of P2 per ml. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Arrows indicate the position of DIRH subunits. The gel was exposed for 16 h at −70°C; molecular mass markers (in kilodaltons) are indicated to the left.

DISCUSSION

In this report we present evidence that an insulin receptor is produced in *D. melanogaster* embryos and in two different cultured *D. melanogaster* cell lines. The *D. melanogaster* receptor, thus far, is the only insulin receptor other than the human whose structure has been analyzed by use of site-specific antibodies and molecular cloning. Since invertebrates and vertebrates diverged over 800 million years ago, it is important to consider conserved and nonconserved features of the *D. melanogaster* and human insulin receptor homologs. In both *D. melanogaster* and mammals, the insulin receptor is a protein tyrosine kinase (17, 19, 27). Partially purified preparations of glycoproteins from both *D. melanogaster* embryos and cultured cell lines catalyze the insulin-dependent phosphorylation of 95- and 170-kDa proteins that are immunoprecipitable by an antipeptide antibody elicited to a conserved β subunit autophosphorylation site of the human insulin receptor (Fig. 1 and 3). The insulin-dependent phosphorylation both in intact cells and in vitro is on tyrosine residues, as indicated by direct analysis (Fig. 4) and by immunoprecipitation with antiphosphotyrosine antibodies (Fig. 2 and 3). The insulin-binding components of the receptor complex can be immunoprecipitated by an antipeptide antibody elicited to the α subunit domain of the human receptor (amino acids 702 to 723). These components are between 100 and 120 kDa in embryos (19) and 120 and 110 kDa, respectively, in Kc and S-2 cells (Fig. 5). The immunological evidence supports conservation of the β-subunit (kinase) domain of the DIRH, as predicted from the genomic sequence (18), and conservation of at least the C-terminal domain of the α subunit of the DIRH. The latter prediction has recently been confirmed in the publication of a partial cDNA sequence of the DIRH (16). Thus the DIRH subunits are similar in molecular mass, function, and primary protein structure to those of the human receptor. Furthermore, like

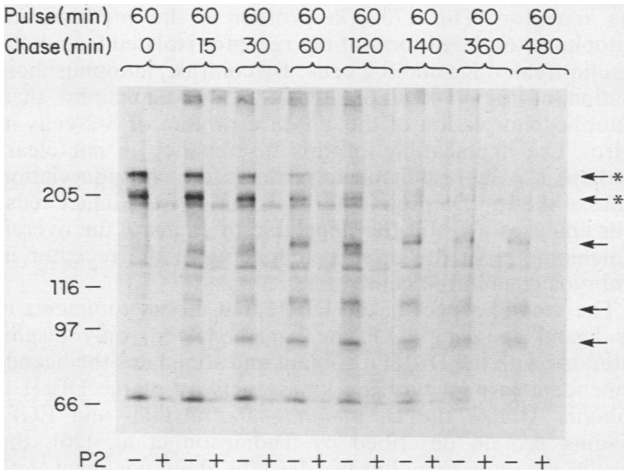

FIG. 11. Pulse-chase analysis of the DIRH. S-2 cells were labeled as described in Materials and Methods for 60 min with [^{35}S]methionine and chased for the periods indicated on top of the figure. The solubilized crude membrane pellet (see Materials and Methods) from each time point was analyzed by immunoprecipitation with AbP2 in the presence or absence of P2. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Molecular mass markers (in kilodaltons) are indicated to the left. Immunoreactive polypeptides are indicated by the arrows to the right. The gel was exposed for 6 days at −70°C.

the human receptor, they are glycoproteins, exposed on the cell surface, held together by disulfide bonds (Fig. 9), and synthesized from a higher-molecular-weight precursor (Fig. 11).

Three aspects of the DIRH structure and physiology may differ from that of the mammalian receptor. First, in Kc cells, and to a lesser extent in embryos and S2 cells, there is a 170-kDa receptor component in addition to the α and β subunit homologs. Evidence that this protein is, in fact, a component of the insulin receptor includes the following: (i) it can be immunoprecipitated (after reduction) by AbP2 and AbP6 but not by antibody to the mammalian EGF receptor; (ii) it is specifically phosphorylated on tyrosine residues when exposed to insulin in intact cells and in vitro; (iii) it is linked to the α subunit of the DIRH by disulfide bonds, since only in the unreduced form is it immunoprecipitated by AbP3; (iv) it is exposed on the outside of the cell like the α and β subunits, as evidenced by cell surface iodination experiments; (v) it does not appear to be an insulin receptor precursor, since its appearance and disappearance in pulse-chase experiments mirrors that of the α and β subunits (Fig. 11). The 170-kDa protein does not appear to bind insulin (Fig. 5) and is not, in its dissociated form, recognized by AbP3. This suggests that it may be an incompletely processed proreceptor molecule that does not exhibit the proper conformation for ligand binding or AbP3 recognition. Certainly, incompletely or improperly processed mammalian insulin proreceptors have been reported not to bind insulin (23). The difference, however, is that in *D. melanogaster* the 170-kDa protein appears to be largely or completely incorporated into the receptor oligomer and to be disposed with the rest of the complex on the cell surface. Although insulin could not be cross-linked specifically to the 170-kDa protein, the latter was phosphorylated on tyrosine in response to the addition of insulin to intact cells or in vitro. Since the 170-kDa protein is linked to the α subunit by disulfide bonds (Fig. 8), it is likely that ligand occupation of an α subunit can induce the conformational change necessary for autophosphorylation of both the 170-kDa protein and the β subunit of the receptor. The 170-kDa protein is the predominant autophosphorylated form of the receptor isolated from both insulin-treated Kc and S-2 cells. By contrast, autophosphorylation of the 95-kDa β subunit is more apparent than autophosphorylation of the 170-kDa protein of S-2 cells in vitro. The explanation for this discrepancy is not clear. Perhaps it is more difficult to preserve the phosphorylation state of the 95-kDa protein during isolation from intact cells. Our analyses are still too imprecise to estimate the overall stoichiometry of the three components of the receptor in embryos or cultured cells.

The second aspect of the DIRH that merits comments is its ligand specificity. Of the ligands tested, only insulin interacts with the DIRH α subunit and stimulates the ligand-dependent protein tyrosine kinase activity of the DIRH β subunit. Unlike the *D. melanogaster* insulin- and EGF-binding protein described by Thompson et al. (26), the insulin receptor homolog we describe does not react with EGF and is not immunoprecipitated by antibody to the human EGF receptor. More interestingly, it does not even appear to interact with the insulinlike ligand IGF-I. Although the 95-kDa protein from human placenta immunoprecipitated by AbP2 is phosphorylated in response to IGF-I and to insulin, perhaps because both human receptors are recognized by this antibody (8), the *D. melanogaster* protein immunoprecipitated by the same antibody responds only to insulin (Fig. 6). This is true even at very high concentrations

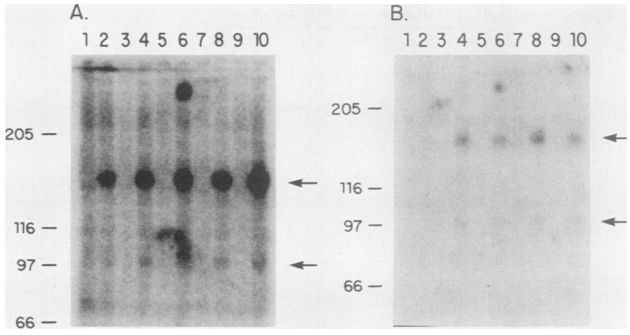

FIG. 12. DIRH phosphorylation in intact cells. Kc cells (A) or S-2 cells (B) were labeled with $^{32}P_i$ and treated for 20 min with either no insulin (lanes 1 and 2) or 2 nM (lanes 3 and 4), 10 nM (lanes 5 and 6), 100 nM (lanes 7 and 8), or 1,000 nM (lanes 9 and 10) insulin. Total cell lysates were solubilized and immunoprecipitated with AbP2 in the absence (even-numbered lanes) or presence (odd-numbered lanes) of P2 (40 μg/ml) and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Each lane in the autoradiogram represents lysate derived from 2×10^6 cells. Molecular mass markers (in kilodaltons) are indicated to the left. The gel was exposed for 5 days at −70°C. Arrows point to the 170-kDa phosphoprotein and the β subunit.

of IGF-I (750 nM), which are able to activate the human insulin receptor. Since the DIRH sequence appears to reflect a single gene in *D. melanogaster* either there is no IGF-I receptor in *D. melanogaster* or it is less similar to the insulin receptor than predicted from the homologies evident in the human proteins. Recently an insulinlike hormone, 4K-PTTH-II, has been purified and completely sequenced (15). Since this protein has two polypeptide chains with amino acid sequence homology to mammalian insulin, as well as conservation of cysteines involved in inter- and intrachain disulfides it was of interest to test its ability to interact with the DIRH. 4K-PTTH-II at 100 nM did not activate the insulin-dependent protein tyrosine kinase activity of the DIRH, although it did stimulate the activity of the human insulin receptor slightly (Fig. 6). Thus it is likely that when the complete structure of *D. melanogaster* insulin is determined, it will be, at least in its receptor-binding domain, more like porcine, bovine, and human insulin than either mammalian IGF-I or silkworm 4K-PTTH II.

A final potential difference between the DIRH and human insulin receptor comes from the evidence that the DIRH is developmentally regulated and that its mRNA is produced maximally during embryogenesis (18, 19). The pattern of insulin receptor expression during early mammalian development is not yet known. It may be that insulin has different functions in embryogenesis and in the mature organism and that genetic analysis in *D. melanogaster* will help us to understand the role of insulin in mammalian development.

In conclusion, a highly specific insulin receptor exists in *D. melanogaster*. Its primary, secondary, and tertiary structures, as well as its functions (ligand binding and ligand-dependent protein tyrosine kinase activity), are remarkably similar to those of the only other insulin receptor studied in similar detail, the human insulin receptor. The presence of this receptor in cultured *D. melanogaster* cell lines and its developmentally regulated production during embryogenesis makes *D. melanogaster* an organism of choice for studying insulin receptor function. It is notable that so far, the insulin receptor is the only tyrosine kinase-linked growth factor

receptor homolog in *D. melanogaster* for which a stimulatory ligand, albeit a mammalian one, is known.

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