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Isolation of a *Drosophila* genomic sequence homologous to the kinase domain of the human insulin receptor and detection of the phosphorylated *Drosophila* receptor with an anti-peptide antibody

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ABSTRACT **A *Drosophila* genomic fragment has been isolated with a deduced amino acid sequence that is strikingly homologous to that of the kinase domain of the human insulin receptor. The *Drosophila* DNA hybridizes with an 11-kilobase mRNA that is most prominent in 8- to 12-hr embryos. An anti-peptide antibody prepared to a sequence in the human insulin receptor kinase domain that is conserved in the *Drosophila* sequence immunoprecipitates a single 95-kDa *Drosophila* protein whose phosphorylation on tyrosine residues is dependent on insulin. We conclude that the DNA sequence is that of the kinase domain of the *Drosophila* insulin receptor and that the 95-kDa phosphoprotein is the autophosphorylated β subunit of that receptor. The results are compatible with our previous reports demonstrating a specific insulin-binding *Drosophila* glycoprotein and an insulin-dependent tyrosine protein kinase whose activity is greatest during embryogenesis. The observations suggest a role for insulin-dependent protein tyrosine phosphorylation during embryogenesis.**

The mammalian insulin receptor is an integral membrane glycoprotein oligomer consisting of two extracellular insulin-binding α subunits (135 kDa) and two β subunits (95 kDa) that span the membrane, contain a cytoplasmic tyrosine protein kinase domain, and are substrates for an insulin-dependent autophosphorylation reaction on tyrosine residues (1–5). All four subunits are held together by disulfide bonds that must be reduced to release the individual subunits (6). The recent cloning of the human insulin proreceptor cDNA (7, 8) that encodes a precursor polypeptide composed of one α and one β subunit confirmed and extended the biochemical evidence that the insulin receptor is a protein tyrosine kinase. Although there is homology between the deduced amino acid sequences of the insulin receptor, the epidermal growth factor (EGF) receptor, and the tyrosine protein kinase oncogenes (7), none of the oncogenes is sufficiently similar to the human insulin receptor to suggest that they are derived therefrom.

Evidence that *Drosophila* possess protein kinases similar to those isolated from mammalian tissues includes the purification to homogeneity of a cAMP-dependent protein kinase (9), identification of genomic sequences homologous to mammalian *src* (10, 11) and *abl* (11), and isolation of DNA sequences homologous to the mammalian EGF receptor (12, 13). Curiously EGF-binding activity has not been detected in *Drosophila* and there is no evidence for a *Drosophila* EGF-like ligand. Sequences related to EGF, however, are present in the Notch gene product (14). In contrast, the existence of an insulin-like molecule in flies and other insects is well-documented (15–17). Furthermore, we have identified a *Drosophila* glycoprotein of 350–400 kDa that binds insulin with high affinity and specificity (18) and contains an insulin-binding component similar in size to that of its mammalian counterpart (18). Although the existence of a tyrosine protein kinase subunit in the putative *Drosophila* insulin receptor was inferred when a developmentally regulated insulin-dependent tyrosine protein kinase activity was detected (19), no direct evidence for the *Drosophila* β subunit was obtained.

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MATERIALS

Restriction endonucleases and DNA polymerase were from New England Biolabs. Calf intestinal phosphatase, total yeast RNA, aprotinin, and soybean trypsin inhibitor were from Boehringer Mannheim. Plasmids were purchased from Promega Biotec (Madison, WI). Nitrocellulose filters and paper were from Schleicher & Schuell. Radiolabeled nucleotides were from Amersham with the exception of dATP-[α-^{35}S], which was purchased from New England Nuclear. The *Drosophila* strain Oregon R was obtained from A. Chovnick.

METHODS

Construction of Human Insulin Receptor Plasmids. Phage containing the human insulin receptor cDNA (7) were partially digested with *Eco*RI and ligated into SP6-5 (20). The plasmid, pLRM, containing the entire 5.2-kilobase (kb) insert was grown on a large scale and isolated according to ref. 21. To construct the β-specific probe, pLRM was digested with *Pst* I and the 1.6-kb fragment was subcloned into *Pst* I-digested pGem 1. The α-subunit-specific probe was prepared by digesting pLRM with *Sph* I and *Pst* I and was ligated into pGem 1.

Screening of the Charon 4A *Drosophila* Genomic Library. The *Drosophila* genomic library was screened according to Benton and Davis (22). The filters were washed and rubbed in 2× SSC (21) and 0.1% $NaDodSO_4$ prior to prehybridization. They were prehybridized for 12 hr at 42°C in 20% formamide/5× SSPE (21)/2× Denhardt's solution (21)/total yeast RNA (0.1 mg/ml), and hybridized in 20% formamide/5× SSPE/2× Denhardt's solution/total yeast RNA (0.1 mg/ml)/10% dextran sulfate/5 ng of nick-translated pLRM DNA per ml (2×10^8 cpm/μg) (23). Filters were washed at 42°C in 2× SSC and 0.1% $NaDodSO_4$ for 1 hr with one change.

Isolation and Subcloning of *Drosophila* Insulin Receptor 18. Clone *Drosophila* insulin receptor 18 was grown and isolated by banding on two sequential CsCl gradients (21). Phage DNA was isolated (21) and digested with *Xho* I and *Eco*RI

Abbreviations: EGF, epidermal growth factor; kb, kilobase(s).

and ligated into pUC19 (24) that was digested with either *Eco*RI or *Eco*RI/*Sal* I.

Preparation of *Drosophila* DNA and RNA. *Drosophila* DNA was isolated according to ref. 25 and transferred as described in ref. 21. Genomic blots were prehybridized with 50% formamide/5× SSPE/2× Denhardt's solution/total yeast RNA (0.1 mg/ml), and hybridized with 50% formamide/5× SSPE/2× Denhardt's solution/total yeast RNA (0.1 mg/ml)/10% dextran sulfate/5 ng of nick-translated *Drosophila* insulin receptor 18 DNA per ml (2×10^8 cpm/μg) (high-stringency conditions).

Staged *Drosophila* poly(A)$^+$ mRNA was isolated, transferred to nitrocellulose, and hybridized as described in ref. 26.

M13 Cloning and DNA Sequencing. *Drosophila* insulin receptor clone 18 was sequenced by the Sanger primer-extension method using dATP[α-^{35}S] (27). Subcloning into either M13 mp18 or M13 mp19 and isolation of the phage for sequencing was performed as described (23). The sequence information obtained by the shotgun method was analyzed with the program of Staden (28).

Immunoprecipitation. *Drosophila* embryo insulin receptor was prepared by chromatography on wheat germ agglutinin lectin (18). Approximately 8–10 fmol of insulin binding activity was incubated in 100 μl containing 50 mM Hepes buffer (pH 7.8), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.5 mM dithiothreitol, 0.25 mCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) in the presence or absence of insulin (4.5 μg/ml) at 23°C for 15 min. ATP was then added to a final concentration of 20 μM and the incubation was continued for 15 min at 23°C. Reactions were stopped by the addition of 10 μl of 200 mM ATP and 4.5 μl of 20% $NaDodSO_4$ and heated at 100°C for 3 min. The samples were diluted 1:10 and incubated with immune or nonimmune serum for 8–10 hr at 4°C. The immune complexes were precipitated with protein A-Sepharose, washed (29), and analyzed by $NaDodSO_4$/PAGE (after heating in the presence or absence of 0.1 M dithiothreitol) in a 7.5% gel (30).

RESULTS AND DISCUSSION

Isolation and Characterization of Genomic Clones. Approximately 60,000 plaques representing >6 genome equivalents from a *Drosophila* genomic library (provided by T. Maniatis) were screened under low-stringency conditions with the human insulin receptor *Aha* III/*Eco*RV fragment (7). This fragment contains sequences encoding both the insulin binding and the kinase domains of the human insulin receptor. Sixteen positive clones were obtained. DNA from each (21) was digested with *Eco*RI, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a probe corresponding to either the α subunit or the β subunit of the human insulin receptor. *Drosophila* insulin receptors 18 and 19 yielded *Eco*RI fragments that hybridized only with the β-subunit-specific probe. The other 14 clones hybridized with the α-subunit-specific probe. The clones were digested with either *Alu* I or *Hae* III and subcloned into *Sma* I-digested M13 mp9. The plaques were transferred to nitrocellulose and those that hybridized with the human probe were isolated and purified.

Partially sequenced inserts (minimally, three sequences per insert) were translated in three reading frames and compared for homology to known proteins using the Dayhoff protein sequence bank (31). *Drosophila* insulin receptor clones 18 and 19 possessed identical translated sequences that were homologous to the human insulin receptor tyrosine protein kinase domain. The other clones had unique restriction patterns and sequences. Since none of these overlapped with *Drosophila* insulin receptors 18 and 19, they will not be further discussed.

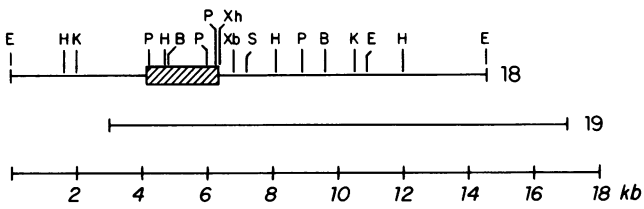

FIG. 1. Restriction map of *Drosophila* insulin receptor genomic clones. Purified phage DNA from *Drosophila* insulin receptors 18 and 19 was digested with *Eco*RI (E), the site of insertion of the fragment into the phage. The *Eco*RI digest was subsequently digested with *Pst* I (P), *Xho* I (Xh), *Bam*HI (B), *Hin*dIII (H), *Xba* I (Xb), *Sac* I (S), and *Kpn* I (K). The sites are indicated above *Drosophila* insulin receptor 18. The hatched box is the fragment sequenced.

A restriction map of *Drosophila* insulin receptor 18 is presented in Fig. 1; it includes the region of overlap with *Drosophila* insulin receptor 19. *Drosophila* insulin receptor clone 18 was digested with *Eco*RI and *Xho* I and subcloned into pUC19, producing the plasmids p18-16, p18-10, and p18-3 containing the 7-kb *Eco*RI/*Xho* I, 5-kb *Eco*RI/*Xho* I, and 3-kb *Eco*RI segments, respectively. Subclone 18-16 contained the region that specifically hybridized to the human probe. This fragment was used for the analysis presented in Fig. 2. The Southern blot suggests that the clone is represented as a single copy in the *Drosophila* genome. Under high-stringency conditions a single large (>17 kb) fragment is seen with *Eco*RI digestion, 1.7- and >17-kb fragments are seen with *Pst* I, and a 6.6-kb fragment is seen with *Hin*dIII. The minor bands may be the result of partial digestion or weakly cross-hybridizing genes. Plasmid p18-16 was digested with *Pst* I, transferred to nitrocellulose, and hybridized with the human cDNA. The 1.7-kb fragment was the only fragment that hybridized (data not shown). It was sequenced and the predicted amino acid sequence of the longest reading frame is presented in Fig. 3. One of the putative autophosphorylation sites of the human insulin receptor is conserved at position 250, as is the consensus ATP binding sequence (32) (residues 79–84). The overall sequence is 53% identical to the deduced amino acid sequence of the human insulin receptor kinase domain (Fig. 4). The greatest similarity extends from amino acid 199 to amino acid 267 of the *Drosophila* sequence (Fig. 3). Here, the receptors are 90%

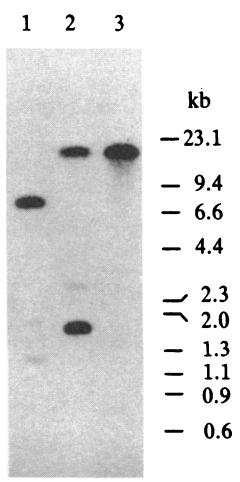

FIG. 2. Southern hybridization of *Drosophila* genomic DNA with *Drosophila* insulin receptor 18 DNA. DNA (5 μg per lane) was digested with either *Hin*dIII (lane 1), *Pst* I (lane 2), or *Eco*RI (lane 3). λ DNA digested with *Hin*dIII and ϕX174 DNA digested with *Hae* III were used as standards. The genomic DNA was prepared from the Oregon R strain of *Drosophila*, whereas the genomic library was made from the Canton S strain.

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      LeuIleGlnGlnProProProSerTyrAlaLysValPhePheTrpLeuLeuGlyIleGly  20
   1  TTAATTCAACAGCCTCCGCCGAGCTATGCTAAGGTCTTTTTCTGGCTACTGGGAATCGGC

      LeuAlaPheLeuIleValSerLeuPheGlyTyrValCysTyrLeuHisLysArgLysVal  40
  61  CTAGCGTTCCTGATCGTTTCCCTGTTCGGCTATGTCTGTTACCTGCACAAGAGGAAGGTT

      ProSerAsnAspLeuHisMetAsnThrGluValAsnProPheTyrAlaSerMetGlnTyr  60
 121  CCCTCTAATGACCTCCATATGAACACAGAGGTGAATCCGTTCTATGCGAGCATGCAATAC

      IleProAspAspTrpGluValLeuArgGluAsnIleIleGlnLeuAlaProLeuGlyGln  80
 181  ATCCCAGACGATTGGGAGGTGCTGCGAGAGAACATCATTCAGTTGGCTCCACTAGGCCAG

      GlySerPheGlyMetValTyrGluGlyIleLeuLysSerProProProAsnGlyValAsp 100
 241  GGATCCTTTGGCATGGTGTATGAGGGTATCCTGAAGTCCTTTCCACCCAATGGCGTGGAT

      ArgGluCysAlaIleLysThrValAsnGluAsnAlaThrAspArgGluArgThrAsnPhe 120
 301  CGCGAGTGTGCCATTAAGACTGTCAACGAAAATGCTACGGATCGGGAGCGAACCAATTTC

      LeuSerGluAlaSerValMetLysGluPheAspThrTyrHisValValArgLeuLeuGly 140
 361  CTGAGCGAGGCGAGCGTCATGAAGGAGTTCGATACGTATCATGTCGTAAGATTGCTCGGT

      ValCysSerArgGlyGlnProAlaLeuValValMetGluLeuMetLysLysGlyAspLeu 160
 421  GTTTGTTCCAGGGGTCAGCCGGCTCTGGTGGTCATGGAGCTAATGAAGAAGGGTGATCTT

      LysSerTyrLeuArgAlaHisArgProGluGluArgAspAspGlyHisAspAspValSer 180
 481  AAGTCCTATTTGCGTGCCCATCGTCCCGAGGAGCGGGATGACGGCCATGATGACGTATCT

      AsnArgIleGlyValThrGlyAsnValGlnProProThrTyrGlyArgIleTyrGlnMet 200
 541  AATCGCATCGGAGTGACTGGTAATGTGCAGCCTCCTACTTATGGAAGAATCTACCAGATG

      AlaIleGluIleAlaAspGlyMetAlaTyrLeuAlaAlaLysLysPheValHisArgPro 220
 601  GCCATTGAGATTGCGGATGGCATGGCATATTTGGCCGCCAAGAAGTTCGTCCATCGTCCC

      PheAlaAlaArgAsnCysMetValAlaAspAspLeuThrValLysIleGlyAspPheGly 240
 661  TTTGCAGCTCGAAATTGCATGGTTGCTGATGATTTGACGGTGAAAATTGGTGACTTTGGA

      MetThrArgAspIleTyrGluThrAspTyrTyrArgLysGlyThrLysGlyLeuLeuPro 260
 721  ATGACCCGTGACATCTATGAGACGGATTACTATCGGAAGGGCACTAAAGGGCTGCTGCCA

      ValArgTrpMetProProGluSerLeuGlnAlaTrpCysLeuLeuLeuValProValThr 280
 781  GTTCGCTGGATGCCACCGGAGAGCTTGCAGGCATGGTGTCTACTCCTAGTGCCAGTGACT

      ValPheSerPheGlyValValLeuTrpGluMetAlaIleLeuSerLeuTrpArgSerPro 300
 841  GTATTCAGCTTTGGAGTGGTTCTCTGGGAAATGGCCATTCTTTCTCTGTGGAGGAGTCCA
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FIG. 3. Nucleotide and deduced amino acid sequence of the *Drosophila* insulin receptor kinase domain. Digestion of subclone 18-16 with *Pst* I yielded a 1.7-kb fragment that was digested and subcloned into M13 mp18 or mp19. The inserts were sequenced (27) and analyzed (28).

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dir   1-LIQQPPPSYAKVFFWLLGIGLAFLIVSLFGYVCYLHKRKVPSNDLHMNTEVNPFYASMQYIPDDWEVL
hir     DYLDVPSNIAKIIIGPLIFVFLFSVVIGSIYL-FLRKRQGPLGPLYASSNPEDVFPCSVYVPDEWEVS
ros

dir     RENIIQLAPLGQGSFGMVYEGILKSFPPNGVDRECAIKTVNENATDRERTNFLSEASVMKEFDTYHVV
hir     REKITLLRELGQGSFGMVYEGNARDIIKGEAETRVAVKTVNESASLRERIEFLNEASVMKGFTCHHVV
ros     RDKLNLHKLLGSGAFGEVYEGTLDILADGSGESRVAVKTLKRGATDQEKSEFLKEAHLMSKFDHPHIL

dir     RLLGVCSRGQPALVVMELMKKGDLKSYLRAHRPEERDDGHDDVSNRIGVTGNVQPPTYGRIYQMAIEI
hir     RLLGVVSKGQPTLVVMELMAHGDLKSYLRSLRP----------EAENNPGRPPPTLQEMIQMAAEI
ros     KLLGVCLLNEPQYLILELMEGGDLLSYLRGARKGKFQSPLLTLTDLLDI----------CLDI

dir     ADGMAYLAAKKFVHRPFAARNCMVADDLTVKIGDFGMTRDIYETDYYRKGTKGLLPVRWMPPESLQAW
hir     ADGMAYLNAKKFVHRDLAARNCMVAHDFTVKIGDFGMTRDIYETDYYRKGGKGLLPVRWMAPESLKD
ros     CKGCVYLEKMRFIHRDLAARNCLVSESRVVKIGDFGLARDIYKNDYYRKRGEGLLPVRWMAPESLID-

dir     CLLLVPVTVFSFGVVLWEMAILSLWRSP
hir     GVFTTSSDMWSFGVVLWEITSLAEQPYQ
ros     GVFTNHSDVWAFGVLVWETLTLGQQPYP
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FIG. 4. Comparison of the deduced amino acid sequence of the *Drosophila* insulin receptor kinase domain with other tyrosine protein kinases. The longest reading frame of the *Drosophila* insulin receptor (dir) sequence was compared to the deduced amino acid sequences of human insulin receptor (hir), v-ros, *Drosophila* abl, the human EGF receptor, fes, fps, and fms. The residues that were homologous to four or more of the sequences are indicated by an asterisk. Identity between *Drosophila* insulin receptor and either human insulin receptor or v-ros is indicated by two dots; conservative nucleotide substitutions are indicated by a single dot.

identical. Next to the human insulin proreceptor, the homology of this region to the deduced amino acid sequence of the v-ros kinase is most striking (60% identical). Homology to the human insulin proreceptor, however, is more significant both here and throughout the kinase domain. The homology to other tyrosine protein kinases including *Drosophila* abl and the *Drosophila* EGF proreceptor homolog is less extensive (see Fig. 4). The sequence to which anti-peptide antibody 2C was prepared is indicated by the solid bar in Fig. 3.

Detection of an mRNA that Specifically Hybridizes to *Drosophila* Insulin Receptor 18 DNA. The probe used for the high-stringency hybridization was a 0.8-kb *Bgl* II/*Bam*HI fragment of *Drosophila* insulin receptor 18-16 containing the region that encodes a portion of the kinase domain. A large mRNA of ≈11 kb hybridized to the probe (Fig. 5). The abundance of this mRNA is greatest between 8 and 12 hr of embryogenesis, coinciding with the biochemical expression of insulin-dependent protein tyrosine kinase activity (19). The size of the mRNA is unexpectedly large if one assumes that the *Drosophila* proreceptor is structurally similar to the human insulin proreceptor (7, 8); the estimated molecular masses of the processed adult *Drosophila* insulin receptor and its insulin-binding subunit are similar to those observed in the mammalian receptor (18, 19). RNA blot analysis of mammalian RNA with the human insulin receptor probe reveals multiple mRNA species, the most prominent of which are ≈8 and 6 kb (7, 8). However, a larger mRNA of ≈11 kb was also noted (8). The mRNAs encoding the v-ros kinase and chicken c-ros are much smaller (33, 34). A less abundant mRNA is also apparent in the female adult, unfertilized eggs, and 0- to 4-hr embryos.

Immunodetection of the β Subunit of the *Drosophila* Insulin Receptor. When the amino acid sequence of the human insulin receptor became available (7, 8), we prepared a panel of anti-peptide antibodies (29). One of these, 2C, was elicited to sequence 1142–1162 of the proreceptor (7). This sequence, T R D I Y E T D Y Y R K G T K G L L P V R, is conserved in the *Drosophila* sequence with the exception of one substitution, G for T at position 14. The homologous sequence in v-ros differs from the human sequence in six positions (33). We reasoned that this antibody might react specifically with the β subunit of the *Drosophila* insulin receptor. As illustrated in Fig. 6, antibody 2C immunoprecipitates one alkali-stable 95-kDa ^{32}P-labeled protein from a *Drosophila* embryo glycoprotein-enriched fraction that had been incubated with [γ-^{32}P]ATP and insulin. Detection is dependent on disulfide reduction and insulin (Fig. 6). The

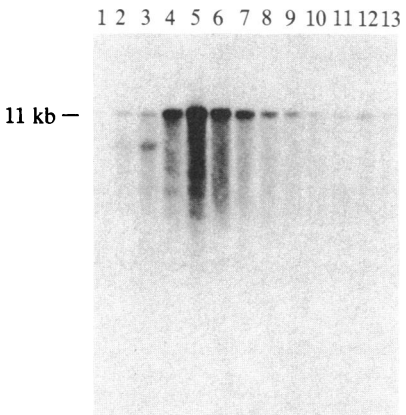

FIG. 5. RNA blot of staged *Drosophila* RNA. Lane 1 is 20 μg of poly(A)$^-$ unfertilized egg RNA; all other lanes are 5 μg of poly(A)$^+$ RNA. Lane 2, unfertilized eggs; lane 3, 0–4 hr; lane 4, 4–8 hr; lane 5, 8–12 hr; lane 6, 12–16 hr; lane 7, 16–20 hr; lane 8, first instar; lane 9, second instar; lane 10, third instar; lane 11, pupae; lane 12, male adult; lane 13, female adult.

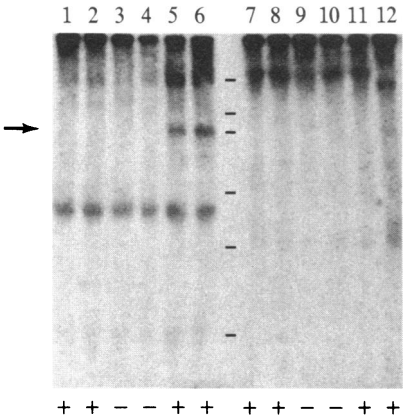

FIG. 6. Phosphorylation and immunoprecipitation of the *Drosophila* insulin receptor. The *Drosophila* insulin receptor was isolated from embryos (0–21 hr) and 1-μg aliquots were phosphorylated in the presence (+) or absence (−) of insulin, immunoprecipitated, and analyzed by NaDodSO$_4$/PAGE under reducing (lanes 1–6) or nonreducing (lanes 7–12) conditions. Lanes 3–6 and 9–12 were immunoprecipitated with antibody 2C and lanes 1, 2, 7, and 8 were precipitated with nonimmune serum. After electrophoresis, the gel was treated with 1 M NaOH at 55°C for 90 min, dried, and exposed to XAR film for 48 hr at −70°C. Bars indicate the positions of molecular size standards. From top to bottom they are 210, 116, 97, 68, 45, and 29 kDa. The arrow indicates the position of the 95-kDa phosphoprotein.

alkaline stability of the phosphoprotein suggested that the phosphate was on tyrosine residues (5). This was confirmed with anti-phosphotyrosine antibody, which immunoprecipitated the protein only when insulin had been present during the autophosphorylation reaction (not shown). The anti-peptide antibody did not immunoprecipitate or immunoblot a v-ros fusion protein (containing the homologous amino acid sequence) or the v-ros kinase expressed in virally infected cells (L. H. Wang, R.H., and O.M.R., unpublished observations).

In conclusion, a *Drosophila* genomic sequence has been cloned that encodes a 300-amino acid sequence strikingly homologous to the kinase domain of the human insulin receptor. The cloned DNA hybridizes to an 11-kb mRNA that is predominantly expressed during embryogenesis consistent with the stage-specific expression of insulin-dependent protein tyrosine kinase activity (19). Support for the conclusion that the isolated DNA encodes the *Drosophila* insulin receptor kinase was obtained by using an anti-peptide antibody to immunoprecipitate an insulin-dependent phosphoprotein of the appropriate size to be the β subunit of the *Drosophila* insulin receptor. The anti-peptide antibody was elicited to a synthetic peptide corresponding to an amino acid sequence of the human receptor that is highly conserved in *Drosophila*. Confirmation will depend on isolation of the transmembrane and insulin-binding domains of the insulin receptor DNA. This is an example of a polypeptide growth factor receptor in *Drosophila* for which there is both biochemical and genetic evidence. It suggests a role for insulin-like polypeptides in early *Drosophila* development.

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