Insulin and related peptides are key hormones for the regulation of growth and metabolism. Here we describe a novel high affinity insulin-related peptide-binding protein (IBP) secreted from cells of the insect Spodoptera frugiperda. This IBP is composed of two Ig-like C2 domains, has a molecular mass of 27 kDa, binds human insulin with an affinity of 70 pM, and inhibits insulin signaling through the insulin receptor. The binding protein also binds insulin-like growth factors I and II, proinsulin, mini-proinsulin, and an insulin analog lacking the last 8 amino acids of the B-chain (des-octa peptide insulin) with high affinity, whereas an insulin analog with a Asp-B10 mutation bound with only 1% of the affinity of human insulin. This binding profile suggests that IBP recognizes a region that is highly conserved in the insulin superfamily but distinct from the classical insulin receptor binding site. The closest homologue of the Spodoptera frugiperda binding protein is the essential gene product IMP-L2, found in Drosophila, where it is implicated in neural and ectodermal development (Garbe, J. C., Yang, E., and Fristrom, J. W. (1993) Development 119, 1237–1250). Here we show that the IMP-L2 protein also binds insulin and related peptides, offering a possible functional explanation to the IMP-L2 null lethality.

The insulin superfamily is an ancient group of structurally homologous proteins, including IGF-I, IGF-II, relaxin, bombxin, and related peptides (1, 2). Insulin-related peptides have been identified in both vertebrates and invertebrates, including mammals, protostomes, nematodes, mollusks, and insects (3–7). Many of these hormones have been shown to act through receptors structurally similar to the insulin receptor, being heterotetrameric, membrane-spanning tyrosine kinases (8, 9). Modulators of this hormone/receptor system have been identified as the IGFs are known to associate with high affinity to a family of circulating binding proteins (IGFBPs) that influence IGF availability and activity (10). In addition, there have been reports of proteins with homology to the IGFBP family that supposedly bind and possibly modulate the activity of insulin (11).

Mammalian insulin is known to exert effects on various insect cell lines (12, 13), and porcine insulin has been demonstrated to bind to an insulin receptor homologue from Drosophila with ~15 nt affinity (14). This receptor has been cloned and demonstrated to be encoded by an essential gene involved in embryonic development both of the cuticle, as well as of the peripheral and central nervous systems (15, 16). Furthermore, the insulin receptor pathway may be involved in controlling cellular metabolism in Drosophila (17).

The Sf9 insect cell expression system has been employed extensively for production of recombinant proteins and has proved to be a good tool for expressing various insulin receptor constructs (18, 19). When trying to express insulin receptor fragments in Sf9 insect cells using a baculovirus system, we discovered insulin binding activity in medium from untransfected Sf9 cells. We were able to track down this binding activity and here describe purification, cloning, and characterization of a novel secreted 27-kDa protein that binds proteins belonging to the insulin superfamily and inhibits insulin activation of the insulin receptor.

EXPERIMENTAL PROCEDURES

Miscellaneous—Human insulin and all insulin analogs as well as 125I-I-(Tyr-A14)insulin were from Novo Nordisk. IGF-II was from GroPeP (Adelaide, Australia). The single-chain insulin/IGF-I hybrid (ICP) consists of insulin with C-domain of IGF-I; detailed description of this construct including purification is found elsewhere (20). Medium for cell cultures, fetal calf serum, Trizol®, and kits for 3′ and 5′-RACE were from Life Technologies, Inc.; DSS was from Pierce; and other chemicals were from Sigma. Insulin-agarose consists of human insulin coupled to Mini-Leak-Beads from Kem-En-Tek (21). Covalent Cross-linking of 125I-Insulin to Sf/IBP—Chemical cross-linking was performed essentially as described (22). Cell culture medium from Sf9 cells was incubated for 60 min at room temperature with 125I-insulin (0.3 nM) in presence or absence of unlabeled insulin (1 μM). Disuccinimidyl suberate (DSS) in dimethyl sulfoxide was added from a 10 mM stock solution to a final concentration of 0.1 mM. After 15 min on ice, the reaction was stopped by adding 0.33 volume of loading buffer containing 100 mM dithiothreitol. Samples were incubated at 70 °C for 10 min before running on a 4–12% polyacrylamide Bis-Tris gel (NuPAGE, Novex). The gel was fixed in 10% acetic acid, 20% ethanol, and a PhosphorImager screen was exposed with the dried gel.

Purification of Sf/IBP—The insulin binding activity was identified in conditioned medium from S. frugiperda (Sf9) cells. The Sf9 cells were grown in Grace’s complete medium containing 2% fetal calf serum, and medium was harvested after 4 days. 2 ml of insulin-agarose was added to 800 ml of conditioned media from the Sf/9 cells and incubated for 18 h at 4 °C with gentle shaking. The insulin-agarose was loaded on a disposable column (Bio-Rad), and sequentially washed with 20 ml of 10
Insulin-binding Protein

**FIG. 1. Covalent cross-linking of 125I-insulin to Sf-IBP protein.** Autoradiogram of 4–12% polyacrylamide gel showing the protein covalently cross-linked to 125I-insulin using 0.2 mM DSS in the absence (−) or presence (+) of 1 μM unlabeled insulin. In adjacent lane was run a 14C-labeled Rainbow marker, with the band sizes indicated at the left of the gel. The following samples were analyzed: conditioned Sf medium (lanes 1 and 2) and control medium (lanes 3 and 4).

**FIG. 2. Purification of Sf-IBP protein from Sf medium.** Coomassie Blue-stained 4–12% polyacrylamide gel. Lane 1, conditioned medium from Sf cells; lane 2, flow-through from insulin column; lane 3, wash 1 (3 mM urea, 0.4 mM NaCl, 0.1 mM citrate (pH 5.0)); lane 4, wash 2 (0.3 mM glycine (pH 3.0)); lane 5, eluate (0.5 mM acetic acid).

mm Tris-Cl, 150 mM NaCl, 20 ml of 3 mM urea, 0.4 mM NaCl, 0.1 mM citrate (pH 5.0), and 0.5 mM glycine (pH 3.0). IBP was eluted with 0.5 mM acetic acid.

**Cloning of Sf-IBP—Purified binding protein was digested with the lysine specific Achromobacter lyticus protease-1, and peptide fragments were separated on NuPAGE running in MES buffer and electroblotted onto polyvinylidene difluoride membrane for amino acid sequencing by standard Edman degradation (ABI 494 protein sequencer, Procise).

In addition, the binding protein was digested with A. lyticus protease-1 and fragments were isolated on an RP-HPLC column and sequenced as above.

Degenerate oligonucleotide primers were designed on the basis of the peptides LF RPMPTK and DTVDDYVPLPK to amplify DNA fragments encoding Sf-IBP from total RNA isolated from Sf cells using Trizol® using the manufacturer's instructions (Fig. 3). After RT-PCR, a combination of these two primers produced a PCR product that after sequencing proved to encode a fragment of Sf-IBP. Specific primers were subsequently designed to allow both 5′- and 3′-RACE to obtain a full-length clone encoding the high affinity insulin-binding protein Sf-IBP.

Using PCR, SF-IBP was equipped with a BamHI site upstream from its native signal sequence and a XbaI site downstream from the coding sequence and inserted into the BamHI/XbaI site of the pZem expression vector. After confirming the full-length sequence, the construct was transfected into BHK cells.

**Expression in Mammalian Cells—**The procedure used for stable expression in cultured BHK cells has been described in detail elsewhere (22). Briefly, the expression vectors were transfected into BHK cells using LipofectAMINE (Life Technologies, Inc.), and, after selection with G418, Briefly, the expression vectors were transfected into BHK cells.

Cloning of IBP-related Protein from Drosophila—The IBP homolog found in Drosophila, IMP-L2 (23), was cloned from a cDNA library (Drosophila, larva 5′-stretch cDNA library from CLONTECH) by PCR using an upstream primer with a BamHI site upstream from the IMP-L2 signal peptide and a downstream primer with a XbaI site after the stop codon. The PCR fragment was digested with BamHI/XbaI and ligated into corresponding site of the pZem vector (24). All sequences were confirmed by sequencing before transfecting into BHK cells.

Ligand Binding Assay—For binding assay, we used the polyethylene glycol precipitation assay. The Sf-IBP and IMP-L2 proteins were obtained from conditioned medium from BHK cells overexpressing these proteins, and Tn-IBP was from conditioned medium from Hi5 cells (from Trichoplusia ni). For the binding assay, a suitable dilution of medium containing binding protein was mixed in a total volume of 200 μl with 5–10 μl 125I-insulin and various concentrations of unlabeled ligand in binding buffer (100 mM Hepes (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 0.05% (w/v) bovine serum albumin, 0.025% (w/v) Triton X-100). After incubation for 16 h at 4 °C, bound counts were recovered by precipitation with 0.2% γ-globulin and 750 μl of 30% (w/v) polyethylene glycol 8000, and bound 125I-insulin was counted in a γ-counter. The concentration of binding protein was adjusted to yield 10–15% binding when no competing ligand was added in the competition assay.

The binding data were fitted to a single binding site model using nonlinear regression algorithm in GraphPad Prism 2.01 (GraphPad Software Inc, San Diego, CA).

**Insulin Receptor Autophosphorylation—**Wheat germ agglutinin-purified recombinant human insulin receptor was incubated in the presence of 10 μM insulin and 10 μM ATP for 45 min at 21 °C and increasing concentrations of purified recombinant Sf-IBP. Phosphoryrosine in the β-subunit of the human insulin receptor was detected by immunoblotting using a horseradish peroxidase-conjugated anti-phosphoryrosine antibody RC20 (Transduction Laboratories). For blotting, samples were mixed with 0.33 volumes of loading buffer containing 100 mM dithiothreitol and incubated at 70 °C for 10 min before loading on a 4–12%
polycrylamide Bis-Tris gel (NuPAGE, Novex). After electrophoresis, proteins were blotted onto Immobilon-P membrane (Millipore) and incubated with RC20 antibody conjugated to peroxidase and finally immunoreactive protein was detected using ECL reagent from Amersham. The Western blot was visualized on a Luminescent Image Analyzer LAS-1000 (Fujifilm) and quantified using Image Gauge software (Fujifilm).

**RESULTS**

Cross-linking of 125I-Labeled Ligand to Sf-IBP—Labeled insulin was chemically cross-linked with DSS to binding protein present in conditioned medium from Sf9 cell cultures, and the gel is shown in Fig. 1. The cross-linking pattern shows specific binding of insulin tracer to a protein with an apparent molecular mass of 30 kDa (binding protein + A-chain of insulin), whereas in the sample from control media no band is seen.

**Purification and Amino Acid Sequencing of Sf-IBP—**Conditioned medium from Sf9 cell cultures was applied to an insulin-agarose column, and after extensive washing, a protein with an apparent molecular mass of 27 kDa was eluted with 0.5 M acetic acid (binding protein + A-chain of insulin), whereas in the sample from control media no band is seen.

**Cloning of Sf-IBP—**Degenerate primers were designed from the peptide sequences of the proteolytic fragments of the purified Sf-IBP protein. mRNA was isolated from Sf9 cells, and, after RT-PCR, several combinations of primers were used to attempt to amplify a fragment of Sf-IBP cDNA. One combination of primers derived from peptides LFRMPTK and DTVDTFVYPLK resulted in a DNA fragment that when sequenced proved to encode a part of Sf-IBP (Fig. 3). Specific primers were designed from this DNA fragment that allowed the cloning of a cDNA encoding full-length Sf-IBP using 3′- and 5′-RACE (Fig. 3).

Performing a similarity search using BLASTP (25) in the Non-Redundant GenBank™ data base revealed that IMP-L2 from *Drosophila* was predicted using SignalP (34). The signal peptide cleavage site is marked with a horizontal arrow, and the four cysteines of the Ig domains are indicated by asterisk. A vertical arrow denotes a N-linked glycosylation site. B, schematic representation of the domain structure of Sf-IBP and IMP-L2.

**Insulin-binding Protein**

The homologous binding protein from *Drosophila*, IMP-L2, was also cloned and overexpressed in BHK cells. The affinity of IMP-L2 for insulin was 81 nM, which is slightly higher affinity than found for IGF-I (0.17 nM) and for IGF-II (0.37 nM). The insulin analogs mini-proinsulin, X145, and ICP all bound to Sf-IBP with higher affinity than insulin, proinsulin bound with similar affinity, whereas X92 and X10 had much poorer affinity than insulin.

The homologous binding protein from *D. melanogaster*, IMP-L2, was also cloned and overexpressed in BHK cells. The affinity of IMP-L2 for insulin was 81 nM, which is a shift of almost 1000-fold as compared with Sf-IBP. This shift in affinity was parallel within the insulin analogs; again, the X145 analog had the highest affinity (17 nM), and the X92 and X10 analogs had low affinities. For the IMP-L2 protein, the IGF-I and IGF-II had slightly higher affinity than insulin, with 17 nM for IGF-I and 42 nM for IGF-II.

In addition to the binding proteins from *S. frugiperda* and *Drosophila*, we also found insulin binding activity in medium from the HI5 cell line that is derived from the cabbage looper, *T. ni*. The initial characterization clearly suggests that the binding activity from the HI5 cells involves a binding protein homologous to Sf-IBP and IMP-L2, and therefore we called it Tn-IBP. This is based on three lines of evidence. First of all, cross-linking experiments show that insulin binds specifically to a 27-kDa protein (data not shown); second, the binding data (Table I) clearly demonstrate high affinity binding of insulin; and, finally, we have several peptide fragment sequences (data

![Amino acid sequence comparison of Sf-IBP and IMP-L2.](http://www.jbc.org/)

**Fig. 4. Amino acid sequence comparison of Sf-IBP and IMP-L2.** A, conserved residues and conservative substitutions are in reversed contrast. The signal peptide cleavage sites in Sf-IBP and IMP-L2 were predicted using SignalP (34). The signal peptide cleavage site is marked with a horizontal arrow, and the four cysteines of the Ig domains are indicated by asterisk. A vertical arrow denotes a N-linked glycosylation site. B, schematic representation of the domain structure of Sf-IBP and IMP-L2.
not shown) that are homologous to the two cloned binding proteins.

\( T_n-IBP \) was found to bind insulin with very high affinity; the \( EC_{50} \) was calculated to be 16 pM, which is about the highest affinity we can estimate with the present assay, and for X145 and proinsulin we observed even higher affinities. The overall picture for insulin and its analogs is the same relative affinities between insulin and its analogs of different biological activity. The affinity of insulin analogs for the human insulin receptor, His-A8, Asp-B10, and Tyr-B25-amide is known to be almost completely inactive in biological assays (27, 28), but for the three insect binding proteins these analogs exhibited affinities similar to or higher than that of insulin, demonstrating that the binding surface involved is quite different from that which binds to the human insulin receptor (29).

**Inhibition of Insulin-stimulated Autophosphorylation of the Insulin Receptor**—When wheat germ agglutinin-purified recombinant human insulin receptor is incubated with insulin and increasing concentrations of \( S_f-IBP \) (purified from conditioned media from transfected BHK cells) a dose-dependent inhibition of insulin receptor autophosphorylation is seen (Fig. 7A). The concentration of \( S_f-IBP \) was estimated from competition binding assays. The inhibition data (quantified on a FujiImager) was fitted to a one-site competition model using nonlinear regression algorithm in GraphPad Prism 2.01. 50% inhibition was seen using \(-9\) nM \( S_f-IBP \), which compares well to the 4 nM insulin used for the autophosphorylation (Fig. 7B).

**DISCUSSION**

When trying to express insulin receptor fragments in \( S_f \) insect cells using a baculovirus system, we discovered insulin binding activity in medium from \( S_f \) cells that had not been transfected. Purification of the binding activity on an insulin-mini-leak column, followed by limited proteolytic digestion and amino acid sequencing of resulting fragments, allowed us to obtain a cDNA encoding a novel insulin-binding protein. This protein was named \( S_f-IBP \) (for \( S.\ frugiperda \) insulin-related peptide-binding protein) and is composed of two Ig-like C2 domains (Fig. 3). Searching for similar proteins in the Non-Redundant GenBank™ data base revealed that IMP-L2 from \( D.\ melanogaster \) had the highest homology (Fig. 4). In addition, \( S_f-IBP \) had homology to three open reading frames from \( C.\ elegans \) encoding secreted proteins with predicted molecular mass of ~27 kDa. The IMP-L2 and one \( C.\ elegans \) gene product were expressed in BHK cells, but only IMP-L2 showed detectable insulin binding. Many other homologous proteins are found in the data base including a number of cell surface receptors, but these are all probably only structurally related due to the Ig-like C2-domains, and not necessarily functionally related.

In addition to \( S.\ frugiperda \) and \( D.\ melanogaster \), we also found insulin binding activity in medium from the \( H_15 \) cell line, which is derived from the cabbage looper, \( T.\ ni \). The initial characterization clearly suggests that the binding activity from the \( T.\ ni \) cells involves a binding protein homologous to \( S_f-IBP \).
and IMP-L2. We have called the T. ni binding protein Tn-IBP.

With the identification of homologous insulin-binding proteins from three different insect organisms, we suggest that insulin-binding proteins containing Ig-like C2 domains may be present in all insects.

The binding data show that Sf-IBP and Tn-IBP have very high affinity for insulin (lower picomolar range), whereas IMP-L2 has an affinity of 80 nM for insulin. When looking at the other insulin-like peptides including the naturally occurring human peptides IGF-I, IGF-II, and proinsulin, as well as the synthetic insulin analogs, the overall picture is that these ligands generally all have affinities close to insulin affinity for the respective binding protein. However, there are a few dramatic deviations from this general pattern; most strikingly, Tn-IBP has approximately 4000-fold poorer affinity for IGF-I and IGF-II than for insulin. The other exception is that insulin analogs with the Asp-B10 mutation (X92 and X10) have lost 10–100-fold in binding affinity.

The synthetic insulin analogs selected for initial characterization of the new insulin-binding protein family are characterized by aberrant behavior in binding to the human insulin receptor. The X92 analog is a superpotent receptor binder (26); the high affinity for the receptor is an additive effect obtained by combining mutations in positions B10 and A8 with deletion of B26–B30 and amidation of Tyr-B25. In contrast, for the IBPs the X92 had lower affinity and the X10 analog demonstrates that the poor affinity is due to the Asp-B10 mutation.

The deleterious effect of the Asp-B10 mutation on Sf-IBP binding and the fact that des-octa peptide insulin (X145) bind with even higher affinity than insulin clearly suggests that Sf-IBP recognize a region of insulin that is highly conserved in the insulin superfamily but distinct from the classical receptor binding site (which involves, among others, the C-terminal B23–B26 region of the insulin B-chain).

When allowed to compete for insulin in an insulin receptor autophosphorylation assay, Sf-IBP acts as an inhibitor of insulin action (Fig. 7). This observation points to a function of Sf-IBP and related proteins in inhibiting or modulating the effect of a putative insect insulin-related peptide.

Searching the recently completed Drosophila genome for insulin-related peptide reveals one cluster of four genes similar to clusters found in Bombyx mori (30) as well as a single insulin-related gene (data not shown). The proteins encoded from these genes share properties with the bombyxins and relaxins in that the C terminus of the B-chain is very divergent from the sequence found in insulin, IGF-I, and IGF-II. The results of our binding studies with des-octa peptide insulin show that this part of the insulin molecule has no importance for its binding to IMP-L2.

IMP-L2 has previously been studied by Fristrom and co-workers (23, 31). IMP-L2 is characterized as being induced by the molting hormone 20-hydroxyecdysone and implicated in epithelial spreading and fusion. Garbe et al. found expression of IMP-L2 in neuronal cells. Genetic analysis demonstrated that IMP-L2 is an essential gene in Drosophila, as disruptions of this gene result in embryonic lethality (23). However, no morphological abnormalities were evident in the embryos, and the authors concluded that they had not identified the cause of lethality in IMP-L2 null Drosophila. Our characterization of IMP-L2 suggests that this protein may be involved in regulating the availability or activity of a Drosophila insulin-like peptide, and that lack of IMP-L2 causes a defective signaling through the Drosophila insulin receptor that in turn leads to the observed lethal phenotype.

Bombyxins are known to stimulate prothoracic glands of the

| Ligand            | Substitutions                              | S. frugiperda | Drosophila | Tricoplusia Ni |
|-------------------|--------------------------------------------|---------------|------------|---------------|
| Insulin           |                                            | 0.07          | 81         | 0.02          |
| Proinsulin        |                                            | 0.02          | 87         | <0.01         |
| IGF-I             |                                            | 0.17          | 17         | 45            |
| IGF-II            |                                            | 0.37          | 42         | 194           |
| Mini-proinsulin   | B29-A1 peptide link                        | 0.04          | 218        | 0.03          |
| X92               | ASH,B10D,B25Y-amide                        | 1.7           | 1270       | 0.15          |
| X137              | B25Y-amide                                 | 0.01          | 94         | 0.01          |
| X10               | B10D                                       | 11            | 2650       | 1.1           |
| X145              | des(B23-B30)                               | <0.01         | 17         | <0.01         |
| ICP               | Insulin w/ IGF-I C-domain                  | 0.04          | 77         | 0.01          |

* Tn-IBP was not cloned, data are based on binding activity in medium from T. Ni cells. Affinities (EC50) were determined from binding curves shown in Figs. 5 and 6.
insect Samia cynthia ricini to synthesize and release the molting hormone ecdysteroid (32). Since 20-hydroxyecdysone induces synthesis of IMP-L2 in Drosophila, it is tempting to speculate that this could provide a regulatory circuit in insect, whereby an insulin-related peptide can regulate the expression of a protein (e.g. IMP-L2) that in turn can regulate the effect of the hormone.

One putative low affinity insulin-binding protein, Mac25, has previously been described (11). This protein was found based on its homology to classical IGF1-binding proteins. Mac25 was shown to bind both insulin IGF-I and IGF-II after recombinant overexpression in insect cell lines (HI5 and S/9). In addition to the domain with homology to IGFBP's, Mac25 also possess a C-terminal Ig-like C2-domain. This domain has very low similarity to the Ig-like C2 domains of S/IBP and IMP-L2. We have expressed this protein with a FLAG tag in S/9 cells to allow easy purification. However, when separated from the S/IBP insulin binding activity, we could not detect any binding of either insulin or IGF-1 to Mac25, suggesting that, if Mac25 binds insulin or IGF-I, the affinity must be below our detection limit of approximately 0.1 μM. Mac25 was also overexpressed in BHK cells with similar negative results on insulin binding (data not shown).

We have searched the NCBI data base for other S/IBP and IMP-L2 homologues, but so far we have only identified three open reading frames from C. elegans that encode secreted proteins composed of two Ig-like C2 domains and a predicted molecular mass around 27 kDa. We have expressed one of these proteins but have not been able to detect insulin binding to this (data not shown). Thus, the possibility remains that this family of IBPs is limited to insects and serves a function that is specific to this class. If a human homologue should exist that is involved in binding proteins belonging to the insulin superfamily, such a protein could be involved in certain disease states (e.g. diabetes or cancer) and as such would represent an obvious and attractive target for drug development.

Acknowledgments—We thank Elisabeth Veyhe Andersen, Else Jost Jensen, and Lene Walander for excellent technical support. We thank the authors, however, reported the affinity for insulin as being ~5 nM, in contrast to our observed affinity of 70 pM. We have no explanation for this discrepancy, but one possible cause could be the difference in binding assay employed. In this study we have used a polyethylene glycol precipitation assay, whereas a charcoal-based assay was used by Doverskog et al. (33).

Addendum—During the preparation of this manuscript, an article appeared describing insulin binding activity in media from S/9 cells (33). This binding activity was characterized by cross-linking and Western ligand blotting as having a molecular weight of approximately 27 kDa and thus appears to be the same binding protein as S/IBP. The authors, however, reported the affinity for insulin as being ~5 nM, in contrast to our observed affinity of 70 pM. We have no explanation for this discrepancy, but one possible cause could be the difference in binding assay employed. In this study we have used a polyethylene glycol precipitation assay, whereas a charcoal-based assay was used by Doverskog et al. (33).

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A New Secreted Insect Protein Belonging to the Immunoglobulin Superfamily Binds Insulin and Related Peptides and Inhibits Their Activities
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