Human Insulin Receptor β-Subunit Transmembrane/Cytoplasmic Domain Expressed in a Baculovirus Expression System: Purification, Characterization, and Polylysine Effects on the Protein Tyrosine Kinase Activity†

Shu-Lian Li, Pei-Fang Yan, I. Benjamin Paz, and Yoko Fujita-Yamaguchi*

Department of Molecular Genetics, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010

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ABSTRACT: We have expressed, purified, and characterized the insulin receptor protein tyrosine kinase (PTK) retaining the transmembrane and downstream domains. The proteins expressed in insect cells using a baculovirus expression system were identified as membrane-bound by immunofluorescence staining and biochemical characterization. One-step purification by immunoaffinity chromatography from Triton X-100 cell extracts resulted in a ~360-fold increase in the specific kinase activity with a yield of ~50%. An appM_r = ~60 000 protein was the major component identified by both silver staining of the purified enzyme and immunostaining of the crude extracts after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Using nondenaturing conditions, the molecular weight was estimated to be ~250 000 and ~500 000 by glycerol gradient centrifugation and gel permeation chromatography, respectively, suggesting that oligomers of the β-subunit domains such as tetramers and octamers are formed. The basal PTK activity of this enzyme was much higher than those of previously reported soluble-form insulin receptor PTKs expressed in insect cells or the native receptor. K_m and V_{max} for two substrates, src-related peptide and poly(Glu, Tyr) (4:1), were 2.4 mM and 2.5 μmol min^{-1} mg^{-1} and 0.26 mM and 1.2 μmol min^{-1} mg^{-1}, respectively. Specific activities measured under two previously reported conditions using histone H2B as a substrate were 100 or 135 nmol min^{-1} mg^{-1}, in contrast to those of soluble PTKs which were reported to be 20 or 70 nmol min^{-1} mg^{-1}, respectively. The purified enzyme was autophosphorylated at Tyr residues. Autophosphorylation activated the enzyme ~3-fold. In the presence of 1 μM polylysine, the kinase activity was stimulated on average ~4-fold. Polylysine treatment concurrently resulted in both aggregation and activation of the enzyme. An oligomeric form of the enzyme with extremely high basal kinase activity and an additional ~4-fold activation and aggregation of the enzyme by polylysine is consistent with the notion that aggregation and concomitant activation of the receptor kinase can occur through the β-subunit domain without participation of the α subunit.

The insulin receptor is a membrane-bound glycoprotein that is composed of two extracellular α subunits and two transmembrane β subunits. The β subunit carries cytoplasmic tyrosine-specific protein kinase (Ullrich et al., 1985; Ebina et al., 1985; Kasuga et al., 1982). The insulin receptor tyrosine protein kinase (PTK)[1] is thought to be responsible for insulin's signal transduction since insulin binding to the extracellular domain of the receptor activates the cytoplasmic PTK and presumably initiates the phosphorylation cascade (Rosen, 1987; Kahn & White, 1988). The PTK activity in vivo and in vitro is regulated by several mechanisms including autophosphorylation (Yu & Czech, 1984; White et al., 1988), Ser phosphorylation (Jacobs & Cuatrecasas, 1986; Bollag et al., 1986; Takayama et al., 1990), and aggregation (Kubar & Obberghen, 1989; Fujita-Yamaguchi et al., 1989a). For example, autophosphorylation, aggregation, and certain polycations (Rosen & Lebwohl, 1988; Fujita-Yamaguchi et al., 1989c; Kohanski, 1989; Morrison et al., 1989) activate PTK activity of the insulin receptor while Ser phosphorylation inhibits PTK activity.

Our previous studies using purified human placental insulin and IGF-I receptors indicated that (i) polylysine activates insulin and IGF-I receptor PTKs (Xu et al., 1991), (ii) receptor aggregation and kinase activation occurred in the presence of polylysine (Xu et al., 1991), (iii) the acidic domain downstream of PTK domains may be responsible for PTK activation by polylysine (Fujita-Yamaguchi et al., 1989b,c), and (IV) insulin receptor aggregates (~8-mer) are much more active as kinases than the monomeric form of $\alpha_2\beta_2$ as judged by radiation inactivation experiments (Fujita-Yamaguchi et al., 1989a).

In order to further investigate how the insulin receptor PTK activity is regulated, it would be beneficial to isolate the PTK domain of the receptor for several reasons. Since the insulin receptor is not only a minor component in cells but also a large multimeric molecule consisting of extracellular binding domains as well as labile cytoplasmic kinase domains, it is difficult to purify sufficient quantities of the intact receptor with full kinase activity (Kathuria et al., 1986; Fujita-Yamaguchi et al., 1989c). The presence of the extracellular binding domain may modulate the kinase reaction. Previous studies indicated that the extracellular domain causes suppression of the PTK activity (Shoelson et al., 1988) and receptor aggregation (Johnson et al., 1988). While our study was in progress, other investigators published purification of soluble forms of the insulin receptor PTK expressed in insect cells (Ellis et al.,

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* To whom correspondence should be addressed. Telephone: (818) 301-8376. FAX: (818) 301-8271.

[1] Abbreviations: PTK, tyrosine protein kinase; IRTMTPK, insulin receptor transmembrane tyrosine-specific protein kinase (β-subunit domain consisting of 871–1343; Ullrich et al., 1985); IGF-I, insulin-like growth factor I; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGF, epidermal growth factor; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; BAEE, *N*,*O*-benzoyl-L-arginine ethyl ester.

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1988; Herrera et al., 1988). In contrast to these studies, we wished to express a membrane-bound PTK since we believe that the transmembrane domain and associated downstream amino acids are crucial for enzyme activity. It is also likely that this domain mediates interactions between the receptor and other membrane-associated molecules which are potentially important for regulating transduction of the insulin signal. Previously, the membrane-bound form has been expressed in mammalian cells, and analyzed at the cellular level (Ellis et al., 1987; Lebwohl et al., 1991). It has, however, never been purifed for further characterization. The baculovirus expression system has been demonstrated to be superior to mammalian expression systems for production and purification of large quantities of mammalian proteins (Summers et al., 1987). Soluble forms of insulin receptor PTKs as well as an intact receptor have successfully been expressed in this system (Ellis et al., 1988; Herrera et al., 1988; Paul et al., 1990).

We designed an insulin receptor PTK cDNA construct which would express 58% of the β-subunit domain, 871–1343 (Ullrich et al., 1985), consisting of the extracellular 47 amino acid residues and the transmembrane and cytoplasmic domains (IRTMTPK), and then expressed this protein in insect cells. The membrane-bound IRTMTPK was solubilized and purified. Characterization of the purified enzyme revealed that receptor aggregation and kinase activation occur with IRTMTPK in a manner similar to that observed with the intact insulin receptor. This provided evidence that the β-subunit domain itself possesses features required for kinase activation and concomitant kinase aggregation.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were obtained from BRL (Gaithersburg, MD). Grace's insect medium was purchased from Gibco (Grand Island, NY). Yeastolate and lactalbumin hydrolysate were from Difco Laboratories (Detroit, MI). Fetal bovine serum was from Irvine Scientific (Santa Ana, CA). A synthetic peptide resembling the tyrosine phosphorylation site of pp60^{src} (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, RR-src peptide) was purchased from Peninsula Laboratories (Belmont, CA). Gentamycin sulfate and amphotericin B, poly(Glu,Tyr) (4:1), polylysine [poly(L-lysine), average M_r = 41 000], phenylmethanesulfonyl fluoride (PMSF), *N*,*O*-benzoyl-L-arginine ethyl ester (BAEE), pepstatin A, aprotinin, and leupeptin were from Sigma (St. Louis, MO). Oligonucleotides were synthesized at the DNA and Peptide Synthesis Facility of the City of Hope. Sf9 cells, a wild virus (AcNPV), and plasmids (pAc373 and pVL941) were kindly provided by Dr. Max Summers, Texas A&M University. cDNA for human insulin receptor was provided by Dr. Axel Ullrich, Max Planck Institute, Martinsried, Germany. Rabbit anti-insulin receptor and rabbit anti-insulin receptor peptide have been previously described (Kathuria et al., 1986). Mouse monoclonal antibody 17A3 which recognizes both insulin and IGF-I receptor PTKs (Morgan & Roth, 1986) was provided by Dr. R. Roth (Stanford University, Stanford, CA). [^{35}S]Met and [γ-^{32}P]ATP were purchased from New England Nuclear (Arlington Heights, IL).

Cell Culture. Sf9 cells were cultured in Grace's insect medium containing 10% heat-inactivated fetal bovine serum, 0.33% yeastolate, 0.33% lactalbumin hydrolysate, gentamycin sulfate (50 μg/mL), and amphotericin B (2.5 μg/mL) at 27 °C in monolayer or suspension cultures as described (Summers et al., 1987). When Sf9 cells provided by Dr. Summers were analyzed by a fluorescence-activated cell sorter, an FACS IV (Becton–Dickinson, Mountain View, CA) equipped with dual lasers (argon and UV), we found the cells contained two populations. We also found that Sf9 cells in population 2 were the cells transfected by AcNPV or recombinant virus. We thus subcloned this type of Sf9 cells and used these cells for expression and purification of IRTMTPK.

Construction of pAcIRTMTPK and pVLIRTMTPK and Preparation of Recombinant Viruses. A *Pst*I/*Pst*I fragment encoding human insulin receptor amino acid sequence 871–1343 (Ullrich et al., 1985) was inserted into a unique *Bam*HI site of pAc373 and PVL941 after ligation with linkers, GATCCGCCATGGGCTGCA and GCCCATGGCG. The insulin receptor β-subunit domain (871–1343; Ullrich et al., 1985) consists of Met followed by a 47 amino acid residue extracellular domain, a transmembrane domain, and a cytoplasmic domain (IRTMTPK). IRTMTPK thus consists of 474 amino acid residues which gives a theoretical molecular weight of 53 399. Sf9 cells (2×10^6 cells in a 25 cm^2 flask) were cotransfected with 1 μg of AcNPV DNA and 2 μg of recombinant plasmid DNA, pAcIRTMTPK or pVLIRTMTPK. Purification of recombinant virus was achieved by four rounds of serial-point dilution and dot hybridization (Fung et al., 1988), using a ^{32}P-labeled insulin receptor *Pst*I/*Pst*I cDNA fragment.

In order to examine the expression of IRTMTPK, Sf9 cells (10^7 cells in a 75 cm^2 flask) were cotransfected by wild-type AcNPV or purified recombinant viruses, washed with PBS and Met-free medium, successively, and plated in a 25 cm^2 flask. The cells were cultured in 1 mL of Met-free medium containing ~0.4 mCi of [^{35}S]Met at 27 °C for 6 h. The cells were harvested, washed with PBS, and lysed in 0.5 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 2% Triton X-100, 2 mM BAEE, 1 mM PMSF, 1 mM EDTA, and 2 μg/mL each of aprotinin, pepstatin A, and leupeptin. The lysates were immunoprecipitated by rabbit anti-insulin receptor β C-terminal peptide or monoclonal antibody 17A3 (1:100 dilution) followed by anti-mouse IgG and Staph A[2] (final 2% suspension). The precipitates were washed and subjected to SDS–PAGE (7.5% gel) and autoradiography. A M_r = ~60 000 protein was expressed when Sf9 cells were infected by either one of the recombinant viruses. Cells untransfected or transfected with a wild-type virus did not produce the M_r = ~60 000 protein. Since the level of expression achieved by VLIRTMTPK virus was not as high as expected when compared to that obtained with AcIRTMTPK virus, we used the AcIRTMTPK recombinant virus for production and purification of IRTMTPK.

Immunostaining of IRTMTPK Expressed in Cells. Sf9 cells were grown on cover glasses and infected by AcIRTMTPK recombinant virus. On day 3 postinfection, cells were fixed by the addition of 0.1 volume of 37% formaldehyde followed by incubation for 20 min at 25 °C. The cells were washed twice with PBS and incubated with 0.2% Triton X-100 containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ for 5 min at 25 °C. The cells were washed twice with PBS and incubated with PBS containing 5% fetal calf serum at 37 °C for 30 min. After the solution was aspirated, the cells were briefly treated with PBS containing 0.2% Triton X-100, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ and incubated with 20 μL of PBS containing 5% fetal calf serum and monoclonal antibody 17A3 for 20 min at 37 °C. The cells were washed 4 times and incubated

[2] Staph A is prepared from lyophilized *S. aureus* cell powder (Sigma P-9151) by washing 9 times with 100 mM Tris-HCl, pH 7, containing 2% SDS and 20 mM DTT and 3 times with PBS (pH 7.2) containing 3% Triton X-100 and 0.1% *N*-sarcosine (Sigma L-5125).

with FITC-labeled anti-mouse IgG for 30 min at 37 °C. After being washed 3 times with PBS, the coverslips were mounted in a 1:1 solution of glycerol and PBS. The cells were analyzed in an Olympus BH2 phase-contrast microscope equipped for epifluorescence. Photographs were taken using Kodak Tri-X pan 400.

Western Blot Analysis of Cell Lysates. Cells infected with AcIRTMTPK recombinant virus were cultured at 27 °C. At different postinfection periods, cells were harvested by centrifugation for 5 min at 200*g*, washed, and lysed in 10 mM ethanolamine, pH 9.8, containing 2% Triton, 10% glycerol, 10 mM EDTA, 1 mM PMSF, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin A. The lysates were subjected to SDS–PAGE (7.5% gel). After electrophoresis, proteins were transferred to a nitrocellulose membrane as described (Towbin et al., 1979). The membrane was incubated with rabbit anti-insulin receptor IgG (1:100 dilution), and the band reacting with the antibody was labeled with ^{125}I-labeled protein A (Kathuria et al., 1986).

Purification of IRTMTPK. Cells (~10^8 cells/100 mL) were infected with AcIRTMTPK recombinant virus and cultured at 27 °C for 3 days in a spinner flask. Cells (~6 × 10^8 cells) were then harvested from three spinner flasks by centrifugation for 5 min at 200*g*, washed twice with PBS, and lysed by freeze–thawing 3 times in 5 mL of 50 mM Tris-HCl, pH 7.4, containing 1 mM PMSF, 2 mM BAEE, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin A (lysis buffer). After the addition of Triton X-100 to a final concentration of 2%, cells were solubilized by stirring at 4 °C for 40 min. These five inhibitors were included in all the buffers during purification. The cell extracts were centrifuged at 100000*g* at 4 °C for 1 h. The supernatant, 9.5 mL, was diluted 4-fold with the lysis buffer (Triton X-100 extracts). An aliquot of the Triton X-100 extracts, 5.5 mL, was incubated with 4.5 mL of 17A3–Sepharose at 4 °C for 16 h with gentle shaking. The gel was packed in a column and washed with 150 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 and 0.15 M NaCl. The column was eluted with 130 mL of 50 mM sodium carbonate buffer, pH 11, containing 0.1% Triton X-100 and 1 M NaCl. Fractions containing PTK activity were combined and concentrated by Centricon 10 (Amicon). The combined and concentrated fractions were used for enzyme assays and protein analysis, respectively. The activity recovered in flow-through fractions was rechromatographed. All the activity which did not bind to the affinity column initially was recovered in the bound fractions from the second 17A3–Sepharose chromatography.

Other Methods. Kinase and autophosphorylation assays (Xu et al., 1991; Sahal et al., 1988), glycerol and sucrose density gradient centrifugation (LeBon et al., 1986; Xu et al., 1991), and phosphoamino acid analysis (Fujita–Yamaguchi et al., 1989c) were performed as previously described.

RESULTS

Time Course of IRTMTPK Expression. Western blot analysis of cell lysates from days 1–5 postinfection indicated that the expression level of IRTMTPK peaked on days 2 and 3 postinfection (Figure 1). Thus, cells were harvested on day 3 postinfection for purification of the protein.

Surface Expression of Recombinant IRTMTPK. Immunofluorescent staining of Sf9 cells with monoclonal antibody 17A3 after infection with the recombinant virus revealed that IRTMTPK expressed in Sf9 cells were membrane-bound (Figure 2). No immunofluorescence was observed when cells

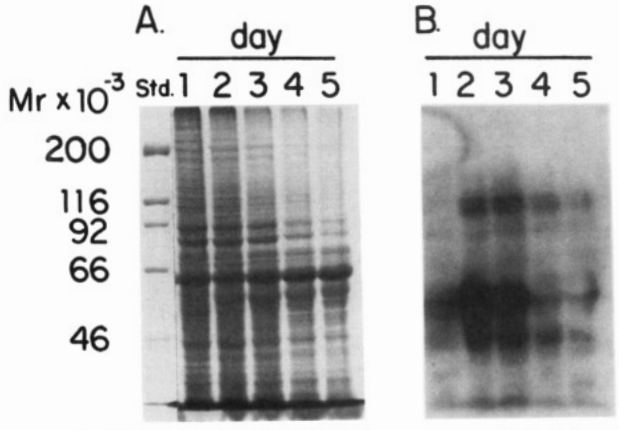

FIGURE 1: Time course of IRTMTPK expression. Cells infected with recombinant virus, AcIRTMTPK, were harvested on day 1–5 postinfection. Cell lysates were analyzed by SDS–PAGE and Western blot using rabbit anti-insulin receptor as described under Experimental Procedures. Shown are a Coomassie-stained gel (A) and an autoradiogram of its Western blot (B).

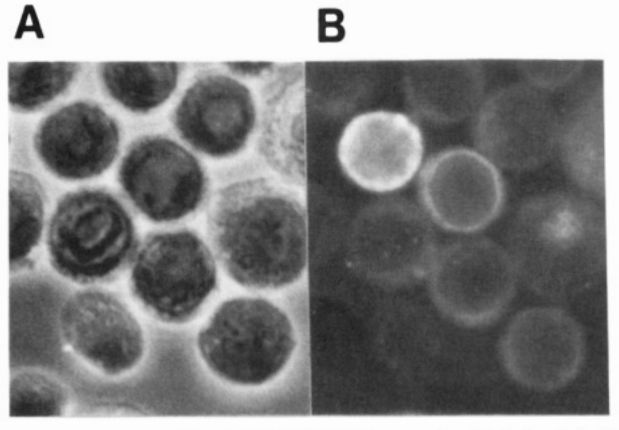

FIGURE 2: Expression of IRTMTPK in insect cells infected with IRTMTPK recombinant virus. Cells grown on a cover glass in a well of a 24-well plate were infected with recombinant virus AcIRTMTPK. On day 3 postinfection, cells were permeabilized and incubated with insulin receptor monoclonal antibody 17A3 (1:100 dilution) followed by FITC-labeled anti-mouse Ig (1:50 dilution) as described under Experimental Procedures. (A) and (B) show cells by phase microscopy and epifluorescence microscopy, respectively.

infected with a wild-type virus were subjected to immunofluorescent staining (data not shown).

To determine subcellular localization of IRTMTPK, cells transfected by AcIRTMTPK recombinant virus were disrupted by freeze–thawing as described under Experimental Procedures divided into two equal portions. One portion was solubilized with Triton X-100 while the other portion was kept without the detergent treatment. The 100000*g* supernatants of these cell homogenates were assayed for PTK activity using RR-src peptide as an exogenous substrate. The PTK activity of the detergent-solubilized preparation which should contain IRTMTPK derived from both cytosolic and membrane-bound fractions was ~30 times higher than that of the cytosolic fraction prepared without the detergent treatment. The result supports that IRTMTPK is a membrane protein.

Purification of IRTMTPK. IRTMTPK expressed in Sf9 cells was solubilized with Triton X-100 and purified by immunoaffinity chromatography using monoclonal antibody 17A3. Eluates with PTK activity were combined and concentrated. Purification is summarized in Table I. Approximately 15 μg of IRTMTPK was purified with a yield of 50% from ~30 mL of cell culture containing ~3 × 10^7 cells.

Table I: Purification of IRTMTPK Expressed in Insect Cells[a]

step	total PTK act. (μmol/min)	yield (%)	total protein (μg)	sp act. (nmol min^{-1} mg^{-1})	purification (x-fold)
Triton X-100 extract	18.1	100	11 000	1.65	1
17A3–Sepharose	9.1	50.2	15.3	595	360

[a] Triton X-100 extracts of the cells transfected (~14 mL of a 100-mL culture containing ~10^8 cells) were subjected to immunoaffinity chromatography using insulin receptor monoclonal antibody 17A3. PTK activity was measured using RR-src peptide as a substrate. Experimental details are described under Experimental Procedures.

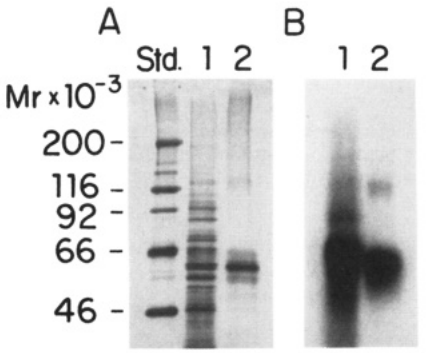

FIGURE 3: SDS–PAGE analysis of purified IRTMTPK and crude extracts from cells infected with recombinant virus AcIRTMTPK. Triton X-100 extracts (0.5 μL) and concentrates from 800 μL of the 17A3–Sepharose eluates shown in Table I were autophosphorylated at 25 °C for 40 min in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100, 40 μM [γ-^{32}P]ATP, 15 mM $MgCl_2$, and 2 mM $MnCl_2$. The reaction was stopped by adding 3× Laemmli's sample buffer (Laemmli, 1970). The samples were applied to a 7.5% SDS–PAGE gel. After electrophoresis, the gel was stained with silver (A) and autoradiographed (B). Lanes 1 and 2 correspond to Triton X-100 extracts and purified IRTMTPK, respectively.

SDS–PAGE analysis of the Triton X-100 extracts and purified IRTMTPK is shown in Figure 3A. A protein band with M_r = ~60 000 was a major component. Minor bands with M_r = ~56 000[3] were also observed.

Autophosphorylation of Crude Extracts and IRTMTPK. Triton X-100 extracts and purified IRTMTPK were subjected to the phosphorylation reaction, SDS–PAGE, and autoradiography. Radioactive bands with M_r = ~60 000 were seen on the autoradiogram (Figure 3B). Phosphoamino acid analysis indicated that both crude and purified enzyme preparations predominantly phosphorylated at Tyr residues of M_r = ~60 000 proteins (Figure 4, lanes 1, 2, and 4).

Enzymatic Analysis. The phosphorylation reaction rate catalyzed by the purified IRTMTPK was linear up to 40 min (Figure 5A). When the time course of prephosphorylated enzyme was compared to that of nonprephosphorylated enzyme, kinase activation was ~3-fold (Figure 5A). The fold increase in kinase activation was not as high as expected. This would be a consequence of the very high basal level of kinase activity as discussed below. Autophosphorylation of insulin receptor PTKs thus far reported exhibited variable degrees of kinase activation: ~100-fold activation with one soluble PTK (see Table II) (Cobb et al., 1989), ~2-fold activation with another soluble PTK (Kallen et al., 1990), and generally a 3–10-fold increase in the activity of purified intact insulin receptor kinase (Kasuga et al., 1983; Pike et al., 1986; Fujita-Yamaguchi et al., 1989c).

[3] These proteins were also detected by Western blot analysis using rabbit anti-insulin receptor IgG (Figure 1B, lanes 2 and 3).

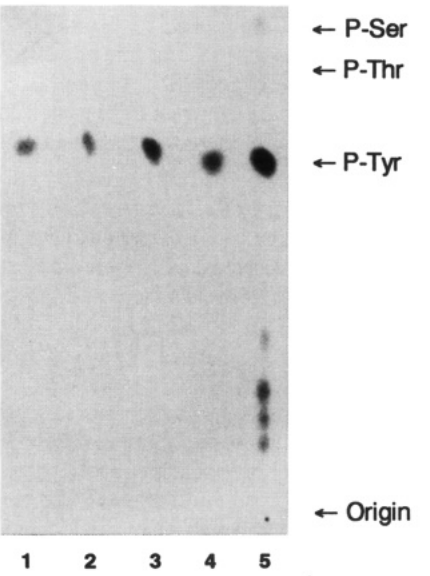

FIGURE 4: Phosphoamino acid analysis of crude and purified IRTMTPK. Radioactive bands corresponding to IRTMTPK as seen in Figure 3B were excised, washed, eluted by trypsin digestion, and hydrolyzed in 6 N HCl for 2 h at 110 °C. Phosphoamino acids were separated by paper electrophoresis at pH 3.5 as described (Perdue et al., 1991). Radioactivity incorporated was visualized by autoradiography. The localization of ninhydrin-stained P-Ser, P-Thr, and P-Tyr is indicated by arrows. Lanes 1 and 4, basal phosphorylation of crude and purified IRTMTPK, respectively; lane 2, insulin-stimulated phosphorylation of crude IRTMTPK; lanes 3 and 5, polylysine-stimulated phosphorylation of crude and purified IRTMTPK.

Kinetic analysis revealed K_m and V_{max} for RR src-peptide and poly(Glu,Tyr) (4:1) of 2.4 mM and 2.5 μmol min^{-1} mg^{-1} (Figure 5B) and 0.26 mM[4] and 1.2 μmol min^{-1} mg^{-1} (data not shown), respectively. The V_{max} of the basal phosphorylation reaction was much higher than that found for the intact receptor; i.e., K_m and V_{max} of purified insulin receptor were 3.7 mM and 100 nM min^{-1} mg^{-1} when assayed at 30 °C using RR-src peptide as a substrate in the presence of 2 mM DTT (Pike et al., 1986). The 25-fold difference in the V_{max} expressed in moles per minute per milligram would be a 6.7-fold difference if the enzyme turnover number is compared based on molecular masses of 60 000 and 225 000 daltons for IRTMTPK and the intact receptor, respectively. The kinase activity assayed under our assay conditions (25 °C and no DTT), however, would be lower than that obtained under their conditions. In fact, DTT treatment on IRTMTPK for 30 min at 25 °C stimulated the PTK activity of IRTMTPK by 1.8-, 3.6-, and 5.2-fold at concentrations of 0.1, 1, and 10 mM DTT, respectively. Thus, IRTMTPK appears at least 10 times more active than the intact insulin receptor PTK.

Since different conditions have been used by different investigators, in order to compare the specific PTK activity of IRTMTPK with those of soluble PTKs previously published, we assayed IRTMTPK using histone H2B as a substrate under two conditions employed by Herrera et al. (1988) and Viallabla et al. (1989), who reported specific activities of 20 and 70 nmol min^{-1} mg^{-1} for their soluble forms of insulin receptor PTK, respectively. Specific activities of our IRTMTPK were 100 and 135 nmol min^{-1} mg^{-1} when assayed under the

[4] Instead of using the average molecular weight of the Tyr polymer, the molarity of Tyr in the polymer was used for expressing their concentrations. (A unit containing one Tyr was considered as unit molecular weight.)

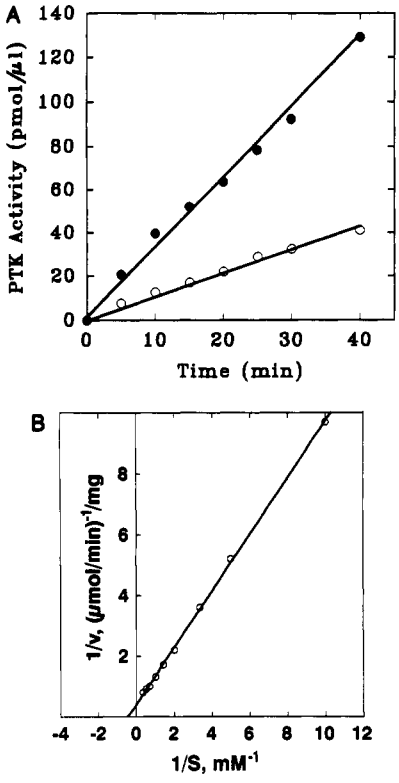

FIGURE 5: Enzymatic characterization of purified IRTMTPK. (A) Time course of enzyme activity: Effect of autophosphorylation. Purified IRTMTPK was prephosphorylated for 30 min at 25 °C as described in Figure 3; then phosphorylation of poly(Glu,Tyr) (4:1) was initiated by adding the polymer substrate (●). Basal phosphorylation of IRTMTPK was initiated by adding the ATP–metal ion mixtures and the polymer substrate (○). At each point, the reaction was stopped by adding 2 mM cold ATP; an aliquot was spotted onto phosphocellulose paper strips (Whatman P-81) and washed in 10 and 5% chilled TCA solution as described (Sahal et al., 1988). (B) Kinetic analysis. The enzyme activity was measured using RR-src peptide as a substrate. Shown is a Lineweaver–Burk plot of the results.

conditions of Herrera et al. (1988) and Viallabla et al. (1989), respectively.

Molecular Weight Estimation. Using SDS–PAGE under reducing and nonreducing conditions, the molecular weight of the IRTMTPK was estimated to be ~60 000 and >200 000, respectively. Under nondenaturing conditions, the molecular weight of the enzyme was estimated to be ~250 000 and ~500 000 by glycerol gradient centrifugation and gel permeation chromatography, respectively. These results suggest that the IRTMTPK expressed in insect cells is not monomeric. Detection of two large molecular forms, M_r = ~250 000 and ~500 000, is consistent with the possibility that the purified IRTMTPK is an octamer composed of two disulfide-linked tetramers.

To examine whether the high molecular weight forms are generated during or after purification, the Triton X-100 extracts were analyzed by Superose 6 chromatography. The eluates were assayed for kinase activity using RR-src peptide as a substrate (Figure 6). The elution profile with closed circles shows a small peak of PTK activity at the void volume, presumably representing IRTMTPK aggregates, and a major peak, which appears to contain at least two components of M_r = ~520 000 and ~250 000. When Superose 6 chromatography was performed at one-fifth of the flow rate by collecting one-fifth of the fraction volume, the PTK activity elution profile revealed many peaks of over M_r = ~250 000 as illustrated by the open circles in Figure 6. These results suggest that the two large molecular weight forms observed with purified IRTMTPK also exist in the crude preparation.

Effects of Polylysine on the PTK Activity of IRTMTPK. Polylysine stimulated the PTK activity of IRTMTPK at concentrations of 0.05–5 μM (Figure 7). At 1 μM polylysine, the kinase was stimulated (4.3 ± 0.7)-fold (n = 3). Phosphoamino acid analysis indicated that polylysine stimulated phosphorylation of both crude and purified IRTMTPK predominantly at Tyr residues of M_r = ~60 000 proteins (Figure 4, lanes 3 and 5).

In order to examine whether polylysine is producing both aggregation and kinase activation of IRTMTPK as observed with purified intact insulin receptors (Xu et al., 1991), sucrose density gradient centrifugation was performed. Figure 8 shows SDS–PAGE analysis of sucrose density gradient centrifugation fractions of IRTMTPK which had been autophosphorylated in the absence (A) or presence (B) of 1 μM polylysine. The results suggest that polylysine stimulated autophosphorylation activity ~2-fold and caused aggregation of IRTMTPKs.

DISCUSSION

This study describes the expression of a membrane-bound form of the PTK domain of the insulin receptor by a baculovirus expression system.[5] Purification was achieved in one step. The specific activity of the purified IRTMTPK was extremely high, ~10 times higher than that of soluble PTKs. In contrast, the level of IRTMTPK expression was approximately one-tenth of that of the soluble PTKs described.

Properties of insulin receptor PTKs expressed in insect cells are summarized in Table II. In terms of expression levels and properties of the purified enzyme, the four soluble insulin receptor PTKs are very similar. Our membrane-bound IRTMTPK is different from those soluble PTKs in several ways. The most intriguing difference was that IRTMTPK exhibited very high basal specific kinase activity. As evidenced by the amount of protein purified and fold purification achieved, the total activity expressed per cell appears to be similar for the soluble PTKs and our membrane-bound PTK. In other words, the IRTMTPK protein is expressed ~10 times less than the soluble PTKs, but its specific activity is ~10 times higher than those of soluble PTKs. It is possible that there is a limit to the total kinase activity that the cell can tolerate or the total amount of proteins that can integrate into the cell membrane.

Since different investigators used variable assay conditions, it is not possible to directly compare specific activities of PTKs. The best way would be to assay all the different forms of purified insulin receptor PTKs under the same conditions at the same time. To compensate for this situation, we assayed our IRTMTPK under two conditions that have been used by others (Herrera et al., 1988; Viallabla et al., 1989). The specific activities of IRTMTPK assayed under former and latter conditions were 100 and 135 nmol min^{-1} mg^{-1}, respectively while that obtained under our assay conditions was 65 nmol min^{-1} mg^{-1}. The major differences among the three assay conditions are concentrations of ATP and metals and the presence or absence of DTT. For example, the assay mixture of Viallabla et al. contained 100 μM ATP and 1 mM DTT, which could result in a higher specific activity.

While K_m values of the purified IRTMTPK for RR-src peptide and poly(Glu,Tyr) (4:1) are comparable to those of

[5] It should be noted that IRTMTPK was expressed as a membrane-bound protein even though our cDNA construct did not include the signal peptide.

Table II: Comparison of Insulin Receptor PTKs Expressed in Insect Cells

protein sequence;[a] no. of amino acids	cellular localization[b]	appM_r	protein[c] (mg)	yield (%)	purification (x-fold)	sp act. (substrate) (nmol min^{-1} mg^{-1})	ref[d]
948–1343; 396	S	48 000	0.4	35	40[e]	20 (histone)	i
941–1343; 403	S	48 000	1.2	55	35	70 (histone)	ii
959–1355; 397	S	48 000	(0.5)		20[e]	0.44 (histone) 45 (histone)[f] 2.3 poly(Glu,Tyr) 274 poly(Glu,Tyr)[f]	iii, iv
946–1303; 358	S	41 000	0.4	13	15[e]	59[g] poly(Glu,Tyr)	v
871–1343; 473	M	60 000	0.05	50	360	595 (RR-src peptide) 850 poly(Glu,Tyr) 135 (histone)	this study

[a] Amino acid numbers from Ullrich et al. (1985) are used. [b] S and M stand for soluble and membrane-bound, respectively. [c] Purified from 100 mL of Sf9 cell culture. [d] References are (i) Herrera et al. (1988), (ii) Viallabla et al. (1989), (iii) Ellis et al. (1987), (iv) Cobb et al. (1989), and (v) Kallen et al. (1990). [e] Calculated from "% of total protein amounts". [f] When prephosphorylated. [g] Units per milligram.

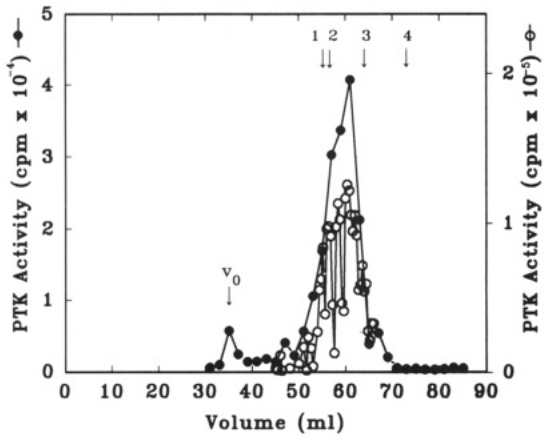

FIGURE 6: Superose 6 chromatography of the Triton X-100 extracts. Approximately 2 mg of the Triton X-100 extracts was applied to a Superose 6 column (1.6 × 50 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100. Fractions were assayed for PTK activity using RR-src peptide as a substrate. Molecular weight markers used are indicated as arrows: (1) bovine thyroglobulin (M_r = 670 000); (2) insulin receptor; (3) bovine γ-globulin (M_r = 158 000); (4) chicken ovalbumin (M_r = 44 000). An initial analysis is shown in (●). The second analysis (○) was performed at one-fifth of the flow rate of the initial analysis by collecting one-fifth of the fraction volume of the initial analysis.

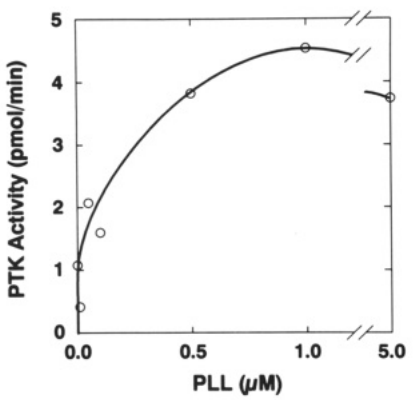

FIGURE 7: Effects of polylysine on the kinase activity of purified IRTMTPK. The immunoaffinity-purified IRTMTPK, equivalent to 26 μL of 17A3–Sepharose eluates, was preincubated with the indicated concentrations of polylysine (PLL) at 15 °C for 20 min and then assayed for PTK activity using RR-src peptide as a substrate as previously described (Xu et al., 1991). Shown are average values of three independent experiments.

the intact insulin receptor (Kasuga et al., 1983; Pike et al., 1986), the V_{max} is at least ~10 times higher than that of the intact receptor. Potential explanations for this high specific activity of IRTMTPK are (i) the constraint of the α subunit, which suppresses PTK activity, is removed (Shoelson et al., 1988), (ii) purification was achieved in one step, resulting in negligible denaturation of the enzyme activity, (iii) IRTMTPK may be modified in such a way that it is a more active form of PTK than the intact receptor (see below), and (iv) IRTMTPK may already be prephosphorylated.

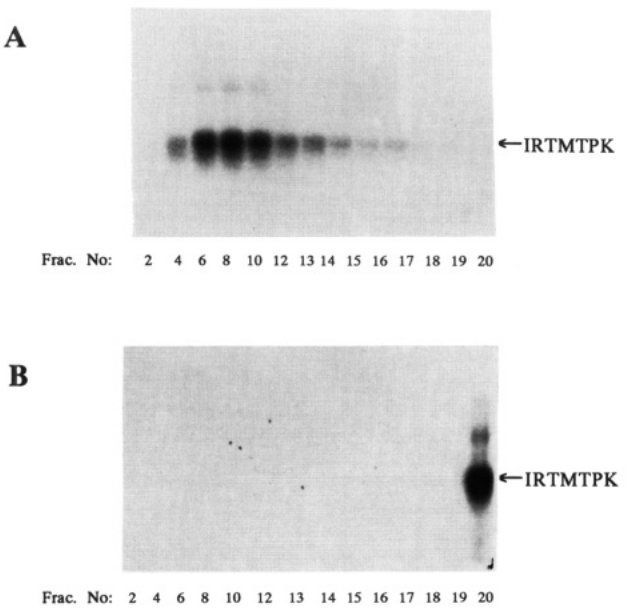

FIGURE 8: Aggregation of IRTMTPKs and activation of autophosphorylation activity caused by polylysine as judged by sucrose density gradient centrifugation. Purified IRTMTPK was preincubated with buffer or 1 μM polylysine at 25 °C for 30 min. Autophosphorylation reaction was then carried out as described in Figure 6. The reaction mixtures were diluted with 50 mM Tris-HCl buffer, pH 7.4, to give a volume of 0.1 mL and layered onto 4.7-mL gradients (5–60%) as described previously (Xu et al., 1991). After centrifugation and collection of fractions of 245 μL each, 10-μL aliquots of the fractions as indicated were subjected to SDS–PAGE (7.5% gel) under reducing conditions. The gels were dried and autoradiographed. Shown are autoradiograms of control (A) and polylysine-treated (B) IRTMTPKs.

As judged by the molecular weight estimated under nondenaturing and denaturing conditions in the absence or presence of DTT, the purified IRTMTPK is an octamer composed of noncovalently associated two disulfide-linked complexes each of which consists of four units of M_r = ~60 000. This oligomeric form may be responsible for the high basal PTK activity observed. We have previously analyzed purified native insulin receptors by radiation inactivation and found that an aggregated form of the receptors, $(\alpha_2\beta_2)_{8-10}$, which is a minor component of purified receptor preparations, exhibits much higher PTK activity than the monomeric $(\alpha_2\beta_2)$ form of the receptor (Fujita-Yamaguchi

et al., 1989a). Thus, the aggregation and concomitant kinase activation described in this study are consistent with previous observations for the intact insulin receptor.

Further structural analysis of the oligomeric form of IRTMTPK is necessary to understand the mechanisms of oligomer formation and kinase activation. Formation of disulfide bonds among four M_r = ~60 000 monomers must involve Cys residues in the cytoplasmic domain of IRTMTPK since only one Cys is available in the extracellular domain of IRTMTPK. It is possible that when solubilized, IRTMTPK may form disulfide bonds which cannot be formed in the intact insulin receptor due to steric hinderence. DTT treatment on IRTMTPK resulted in activation of PTK activity, which could be caused by reduction of SH in the active site of the enzyme. Whether further disruption of the oligomeric structure leads to inactivation of IRTMTPK remains to be answered.

A membrane-bound insulin receptor kinase domain has previously been expressed in Chinese hamster ovary (CHO) cells by Ellis et al. (1987). This kinase domain, designated hIR.spBam, included the signal peptide, the first 12 amino acids of the α subunit, the carboxy-terminal ~20% of the α subunit, and the entire β subunit. A membrane-bound protein with M_r = ~116 000 was expressed, which indicated that proteolytic processing did not take place. Autophosphorylation of the hIR.spBam was observed in an insulin-independent manner. The observed autophosphorylation was ~$^1/_{20}$th of that of a soluble insulin receptor kinase domain expressed in CHO cells or ~$^1/_3$rd of that of the basal autophosphorylation of an intact receptor expressed in CHO cells. These data are in contrast to our observation that the membrane-bound kinase is much more active than soluble forms of the kinase or the intact receptor kinase. Since they did not present the biochemical characterization of their kinases, it is not possible to compare the data closely. The contrasting observation is most likely due to the additional extracellular domain of 322 amino acids in their protein which may have suppressed PTK activity. Of interest, however, is their observation that hIR.spBam mediates a constitutively elevated insulin-independent uptake of 2-deoxyglucose. This observation in conjunction with our data showing IRTMTPK to be a highly active kinase would provide a good correlation between kinase activation and signal transduction.

Another different membrane-anchored insulin receptor kinase (MARK) has been expressed in mouse L cells by Lebwohl et al. (1990). An insulin receptor cDNA consisting of a 27 amino acid signal peptide, a 15 amino acid extracellular domain, a 23 amino acid transmembrane, and a 403 amino acid kinase domain was placed under the transcriptional control of an inducible promoter. They showed that deoxyglucose uptake was enhanced when MARK was induced. Enzymatic characterization of MARK, however, was not presented, which would not allow the comparison of MARK with our IRTMTPK.

A number of studies suggested that aggregation of cell-surface receptors plays an essential role in transmembrane signaling (Kahn et al., 1978; Maxfield et al., 1978; Schlessinger et al., 1978; Schechter et al., 1979; Schreiber et al., 1981, 1983). Cytochemical observations indicated that microaggregation and patching of the receptors were induced by ligands such as insulin and EGF (Kahn et al., 1978; Maxfield et al., 1978; Schlessinger et al., 1978). Anti-receptor antibodies are able to mimic biological effects of the hormone (Kahn et al., 1978; Maxfield et al., 1978; Schlessinger et al., 1978; Schreiber et al., 1981). Similar aggregation phenomena and kinase activation have been observed with the soluble insulin receptor as well (Heffetz & Zick, 1986; Kubar & Obberghen, 1989; Fujita-Yamaghchi et al., 1989a; Kohanski, 1989; Biener & Zick, 1990; Xu et al., 1991). It is thus important to investigate directly the correlation between receptor aggregation and kinase activation. Our present study supports the contention that the greater the degree of aggregation (tetramer, octamer, or even larger aggregates induced by polylysine), the greater the observed specific kinase activity. Since microaggregation induced by anti-receptor antibody might only result in dimerization, it will be necessary to closely compare our observations with previous studies.

We have previously suggested that acidic domains in the insulin receptor may directly interact with polylysine (Fujita-Yamaghchi et al., 1989b,c). These domains are α633–645 (A-I), α695–707 (A-II), and β1268–1286 (A-III). Among these, A-III is the domain which we suggested to be important for the direct interaction with polylysine. When treated with polylysine, IRTMTPK was activated on average 4-fold whereas the intact receptor kinase was activated 10–60-fold (Fujita-Yamaghchi et al., 1989c). This indicates that although not as effectively activated as the intact receptor kinase, IRTMTPK can be activated by polylysine possibly through interaction with the A-III domain since it contains only this domain. To conclusively prove this hypothesis, however, the effect of polylysine on IRTMTPK in which the A-III domain is deleted should be performed. This is now under investigation in our laboratory. The reason that polylysine affects IRTMTPK less potently than an intact receptor is most likely due to the highly activated basal state of the oligomeric IRTMTPK.

We have confirmed that polylysine produced both aggregation and PTK activation of IRTMTPK as judged by sucrose gradient centrifugation. This is a similar phenomenon observed with intact insulin and IGF-I receptors (Kohanski, 1989; Xu et al., 1991). Thus, we have demonstrated that polylysine effects observed with intact receptors can be reproduced using a domain of the β subunit. Construction of additional recombinant insulin receptor PTKs as described here would provide further useful information on structure–function relationships of the receptor kinase.

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Registry No. IRTMTPK, 88201-45-0; PTK, 9004-10-8; polylysine, 25104-18-1; polylysine, SRU, 38000-06-5.