

Efficient Expression in Insect Cells of a Soluble, Active Human Insulin Receptor Protein-Tyrosine Kinase Domain by Use of a Baculovirus Vector

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The human insulin receptor (IR) is a transmembrane glycoprotein, whose cytoplasmic domain contains an insulin-activated protein-tyrosine kinase (EC 2.7.2.112). By the use of an appropriately engineered baculovirus expression vector, a soluble cytoplasmic derivative of this domain was expressed in the insect cell line *Spodoptera frugiperda* (Sf9). At 24 to 48 h after Sf9 cells were infected with recombinant virus, a protein of the size expected for this domain (~48 kilodaltons) constituted a major band when total cell lysates of metabolically labeled cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. This protein (designated AchIRPTK) was immunoprecipitated by three monoclonal antibodies, each of which recognizes a distinct antigenic site of the IR cytoplasmic domain and requires the native structure of the protein for recognition and one of which binds at or near the physiologically relevant site(s) of IR autophosphorylation. In vivo, AchIRPTK was phosphorylated on both tyrosine and serine residues. When affinity purified, the kinase was active in vitro; it autophosphorylated exclusively on tyrosine residues, and phosphorylated the exogenous substrates histone H2b and poly(Glu-Tyr). The expression of an active IR protein-tyrosine kinase molecule in this heterologous cell system provides an efficient experimental method for producing this domain in quantity for enzymatic and structural studies.

The insulin receptor (IR) is a transmembrane glycoprotein composed of two α (735 amino acids; M_r 84,214) and two β (620 amino acids; M_r 69,703) subunits (for reviews, see references 9 and 15). The deduced amino acid sequence of the molecule reveals the overall topology of the receptor, which consists of a large extracellular domain (929 amino acids; all of the α subunit and approximately one-third of the β subunit) that binds the hormone, a single transmembrane domain (23 amino acids), and a cytoplasmic domain (403 amino acids) that contains the insulin-activated protein-tyrosine kinase (PTK; EC 2.7.1.112; 2, 21). Thus the receptor is composed of two large domains, each with a distinct function (insulin binding or PTK activity), that must interact to initiate the physiological responses to the hormone.

We wish ultimately to understand the details of the function of IR domains at the molecular and structural levels. Given the size (1,355 amino acids) of the native integral membrane protein, it would be experimentally advantageous to generate and study these major functional domains as soluble molecules. Our previous studies have demonstrated that a functional wild-type human insulin receptor (hIR), as well as a variety of hIR mutant proteins, can be expressed from cloned cDNAs in mammalian (rodent CHO) cells under the transcriptional control of the simian virus 40 early promoter (3, 5-7; for a review, see reference 4). In the present study, we explored the feasibility of expressing the hIR PTK domain by the use of a vector based on a baculovirus (*Autographa californica* mononuclear polyhedrosis virus [AcMNPV]) in insect (*Spodoptera frugiperda*; Sf9) cells (for a review, see reference 20). This system exploits the very strong polyhedrin promoter late in a lytic

viral infection and can produce large quantities of protein (at levels of milligrams per liter) in cultured insect (Sf9) cells. Furthermore, a number of native functional mammalian proteins have been produced by these methods, a fact that demonstrates the utility of this approach (12, 18, 19; for a review, see reference 20). In contrast, our efforts to express the molecule in bacteria have resulted in the production of only limited quantities of inactive kinase (unpublished observations). With the present study, we demonstrate that a functional hIR PTK can be synthesized efficiently in Sf9 cells by the use of an appropriately engineered baculovirus expression vector.

MATERIALS AND METHODS

Construction of recombinant baculovirus expression vectors. All manipulations of DNA were according to standard procedures (11). Enzymes were from New England Biolabs, Inc., or Boehringer Mannheim Biochemicals. Plasmids were propagated in *Escherichia coli* DH1 as described earlier (8).

The DNA sequence 5' to the initiation methionine codon (AUG) of the polyhedrin gene is strikingly AT rich, in contrast to sequences flanking many mammalian AUGs (cf. CC(A or G)CCAUGG [10] and Fig. 1). For these and subsequent experiments, we wished to utilize the polyhedrin leader and AUG and thus avoid the introduction of extraneous nucleotides (particularly GC-rich sequences, derived from either 5' untranslated or linker sequences) between the promoter, the AUG codon, and convenient cloning sites. Therefore, we first constructed a derivative (pAc373.2) of one of the original baculovirus (AcMNPV) expression vectors (pAc373 [20]) by introducing a short linker sequence

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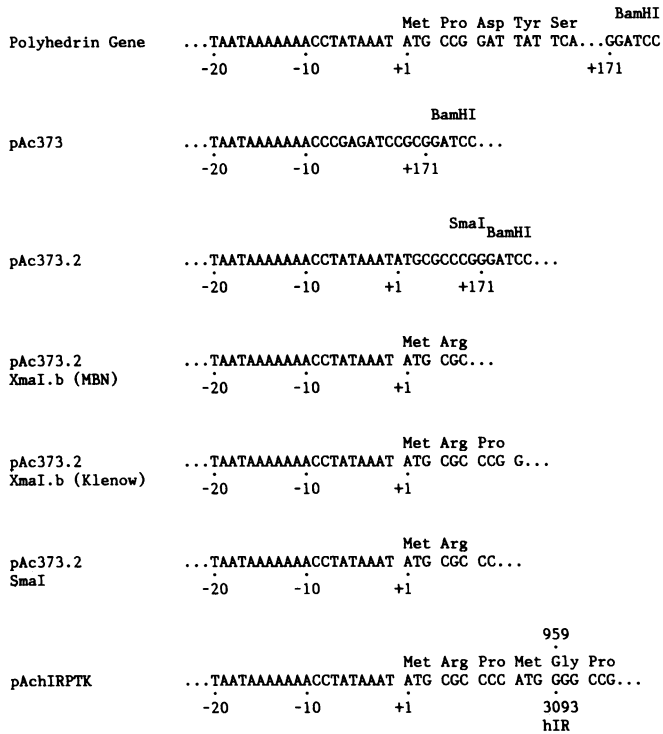


FIG. 1. Nucleotide sequences of relevant regions of vectors utilized in the present study (see Materials and Methods). MBN, Mung bean nuclease. The sequences of the polyhedrin gene and of pAc373 are as described by Summers and Smith (20). The sequences of the indicated regions of pAc373.2 and pAchIRPTK were determined in the present study.

with a *SmaI* (*XmaI*) site between the codon for the initiator methionine at base pair (bp) +1 and the *BamHI* site at bp +171 of the polyhedrin gene. The expression vector pAc373.2 was generated by first deleting the ~100-bp *EcoRV* (bp -96)-to-*BamHI* (bp +171) fragment (containing the polyhedrin 5' untranslated region to bp -8 and a *BamHI* linker [20]; Fig. 1) from pAc373 (generously provided by Max Summers, Texas A & M University, College Station). This fragment was replaced by an ~100-bp *EcoRV*-to-*BamHI* fragment (containing the complete polyhedrin 5' untranslated region, the polyhedrin initiation methionine codon, and *SmaI-XmaI* and *BamHI* cloning sites; Fig. 1) derived from pVL106, an expression plasmid (generously provided by Verne Luckow, Texas A & M University) with *SmaI-XmaI* sites flanking the unique *BamHI* site. This new plasmid, designated pAc373.2 (~9.8 kbp), thus contained the complete polyhedrin 5' untranslated region, initiation methionine codon, and unique *SmaI-XmaI* and *BamHI* cloning sites. Insertion of cDNAs encoding foreign proteins in frame following the polyhedrin AUG (thus with the polyhedrin leader intact) was done by utilizing either the unique 5' *SmaI* or *XmaI* site to adjust the reading frame of the insert cDNA and the unique 3' *BamHI* site (Fig. 1). The *XmaI*-digested vector is rendered blunt ended by the use of either the large fragment of *E. coli* DNA polymerase (Klenow) to fill in the 5' *XmaI* overhang or mung bean nuclease to remove the 5' *XmaI* overhang (Fig. 1).

The cytoplasmic domain of the hIR β subunit, which lacks only the first six residues (RKRQPD) after the transmem-

brane domain, was provided by an ~1.6-kbp *BglII* (bp 3087; residue Gly-959)-to-*HindIII* (provided by a polylinker 3' to the end of the hIR cDNA) fragment as follows. Plasmid pspBam (5) was digested with *BglII* (which left a 3' overhang), rendered blunt ended by the use of Klenow as described elsewhere (3; the flush-end *BglII* site was then in frame with the codon for Gly-959 of the hIR protein), and purified by electrophoresis in low-gelling-temperature agarose (SeaPlaque; FMC Corp., Rockland, Maine). The hIR fragment was then cloned into the bacterial expression vector pKK233.2 (1; Pharmacia Fine Chemicals, Piscataway, N.J.) in frame with a bacterial initiator methionine (included within an *NcoI* site; CCATGG) as follows. (i) pKK233.2 was digested with *NcoI*; the 5' overhang was filled in (to become blunt ended, with the methionine of the ATG codon in frame) by the use of Klenow in the presence of all four deoxynucleotide triphosphates (P-L Biochemicals, Inc. Milwaukee, Wis.) and digested with *HindIII* (at a unique site 3' to ATG). (ii) This 5'-blunt-ended, 3'-*HindIII*-digested vector was ligated to the 5'-*BglII*-blunt-ended, 3'-*HindIII*-digested ~1.6-kbp hIR fragment and used to transform *E. coli* DH1 (8). The hIR insert was subsequently excised from this plasmid (designated pAchIRPTK) by (i) digestion with *NcoI*, (ii) filling the 5' overhang with Klenow and deoxynucleotide triphosphates, (iii) digestion with *BamHI* (provided by a polylinker site 3' to the end of hIR cDNA), and (iv) purification of the insert on low-gelling-temperature agarose. The 5' sequence of this fragment then had an extra base (C) 5' to the methionine codon (ATG) derived from pKK233.2, with the methionine codon in frame 3' with codon Gly-959 (GGG) of the hIR cytoplasmic domain.

Plasmid pAc373.2 (see above) was digested with *SmaI* (which left an arginine codon [CGG] and two extra bases [CC] after the polyhedrin 5' untranslated region and initiator methionine codon [ATG]) and *BamHI*, ligated with the above-described hIR fragment, and used to transform DH1 cells. The resulting plasmid (designated pAchIRPTK) thus encoded (after the polyhedrin 5' untranslated region) the polyhedrin initiator methionine, two heterologous amino acids (Arg and Pro) derived from polylinker sequence, the methionine derived from the *NcoI* site of the bacterial expression vector, and residues 959 to 1355 of the hIR protein (i.e., MRPMG... hIRPTK; compare with ...RKRQPDG... hIRPTK for the wild-type hIR cytoplasmic domain [2]; Fig. 1). That the nucleotide sequences of both pAc373.2 and pAchIRPTK were in fact as described was verified by cloning appropriate fragments into M13 mp18 and mp19 (22) and determining the relevant sequences on both strands (17).

Isolation and purification of recombinant virus and expression of proteins in insect Sf9 cells. All experimental methods for the use of insect (Sf9) cells and viruses were according to Summers and Smith (20). Sf9 cells were generously provided by Max Summers and colleagues (Texas A & M University).

Plasmid pAchIRPTK, together with wild-type AcMNPV DNA, was introduced into cultured Sf9 cells as a calcium phosphate precipitate. Recombinant virus was identified and purified from a viral stock from the transfection by three rounds of plaque purification. Ninety-six putative recombinant plaques were first identified by their occlusion-minus phenotype. Southern blots of crude DNA preparations from Sf9 cells infected with these primary stocks were hybridized with a probe encompassing the hIR PTK domain. Of the positive preparations, the strongest (no. 75) was selected for further analysis. After two further rounds of plaque purifi-

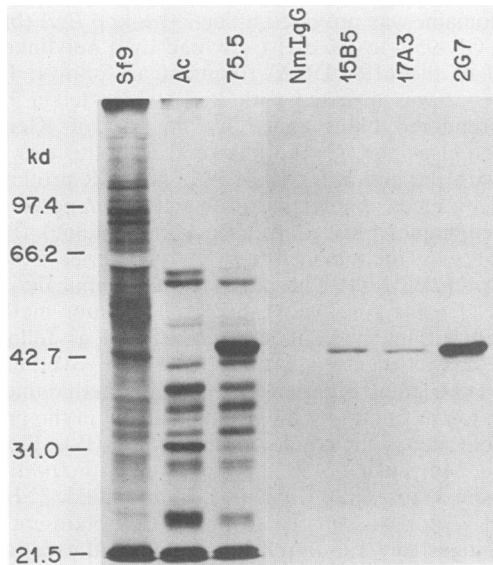


FIG. 2. Identification of hIR PTK domain expressed in metabolically labeled (L - ^{35}S)methionine and L - ^{35}S)cysteine Sf9 cells at 48 h after infection with recombinant virus AchIRPTK 75.5. The first three lanes show total cell lysates (1% of total volume) prepared from mock-infected (Sf9), wild-type-virus-infected (Ac), or recombinant-virus-infected (75.5) Sf9 cells. The last four lanes show immunoprecipitates of lysates prepared from cells infected with AchIRPTK 75.5 virus. NmIgG, Control normal mouse IgG; 15B5, 17A3, and 2G7, MAbs to distinct antigenic sites of the cytoplasmic (including PTK) domain of the IR protein (13, 14). Analysis was by SDS-PAGE and fluorography. The molecular masses of standard proteins are indicated at the left of this and subsequent figures. kd, Kilodalton.

cation, six independent occlusion-minus plaques (designated AchIRPTK 75.1 to AchIRPTK 75.6) were selected. DNA was purified from cells infected with each virus and digested with *Nco*I and *Bam*HI (at unique sites in pAchIRPTK which liberated the hIR PTK insert), and a Southern blot of these DNAs was hybridized with a hIR PTK probe. All six DNAs exhibited the single expected ~ 1.6 -kbp band, which was not present on blots of wild-type AcMNPV DNA. One of these six recombinant viruses, designated AchIRPTK 75.5, was used for the experiments described herein.

Immunoprecipitations and kinase assays of the hIR PTK domain expressed in Sf9 cells were done by the methods described earlier (3). Phosphoamino acid analysis was performed as described elsewhere (16). For biochemical analysis of Sf9 cells, cells were either lysed in buffer plus nonionic detergent (1% Triton X-100; 3) or swollen in hypotonic buffer and homogenized (12). Identical results were obtained with both protocols (see Results and Discussion).

RESULTS AND DISCUSSION

hIR PTK domain expressed in infected Sf9 cells. To examine the expression of the hIR PTK domain, Sf9 cells were either mock infected or infected with wild-type AcMNPV or recombinant virus AchIRPTK 75.5 (see Materials and Methods). At 24 or 48 h postinfection, cells were metabolically labeled for 4 to 6 h with L - ^{35}S)methionine and L - ^{35}S)cysteine. Examination of total cell lysates by sodium dode-

cyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography revealed an ~ 48 -kilodalton (kDa) band in cells infected with AchIRPTK 75.5 but not in wild-type-AcMNPV- or mock-infected cells (Fig. 2). Similar results were obtained with cells infected with each of the recombinant viruses in the series AchIRPTK 75.1 to AchIRPTK 75.6 (data not shown).

To determine whether this ~ 48 -kDa protein expressed in AchIRPTK 75.5-infected cells was indeed hIR PTK, cells were labeled metabolically at 48 h postinfection as described above, and cell lysates were incubated with a panel of monoclonal antibodies (MAbs) specific to the cytoplasmic domain of the IR (generously provided by Richard Roth, Stanford University, Stanford, Calif.; 13, 14). From lysates prepared from AchIRPTK 75.5-infected Sf9 cells (Fig. 2), but not from mock-infected or wild-type-AcMNPV-infected cells (data not shown), the three IR-specific MAbs immunoprecipitated a single band of ~ 48 kDa (Fig. 2, lanes 5 to 7), which was not present when control normal mouse immunoglobulin G (IgG) was employed (Fig. 2, lane 4). Because all three IR-specific MAbs require the native conformation of the protein for recognition and because the three MAbs are each directed to a distinct epitope in the IR cytoplasmic domain (one of which [17A3] binds at or near physiologically relevant site[s] of IR autophosphorylation), these results suggest that the molecule, as expressed in this heterologous cell system, is folded into a conformation which must approximate that of the wild-type hIR.

The pAchIRPTK plasmid was engineered to express the hIR cytoplasmic domain as a soluble protein in the cyto-

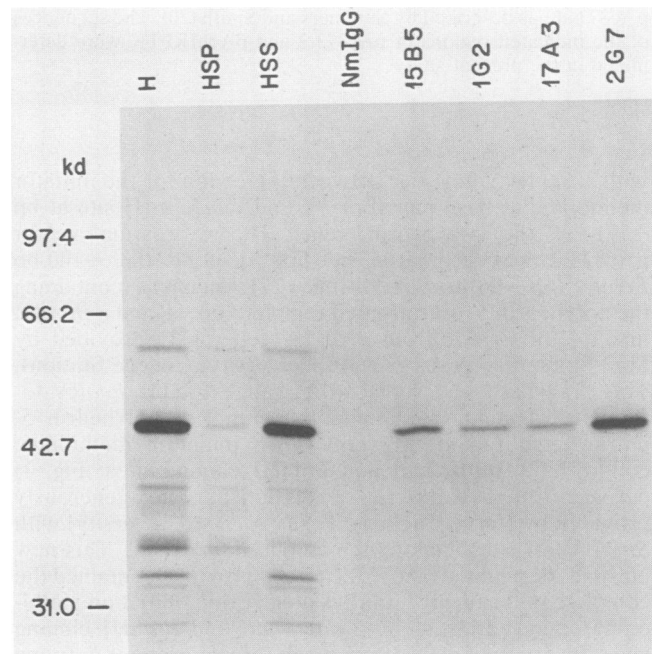


FIG. 3. Subcellular fractionation of metabolically labeled (L - ^{35}S)methionine and L - ^{35}S)cysteine Sf9 cells at 48 h after infection with recombinant AchIRPTK 75.5 virus. The first three lanes show 1% of the total volume of each of the following fractions: total cell homogenate (H), high-speed pellet (HSP), and high-speed supernatant (HSS). The last five lanes show immunoprecipitates of lysates prepared from cells infected with AchIRPTK 75.5 virus. NmIgG, Control normal mouse IgG; 15B5, 1G2, 17A3, and 2G7, MAbs to distinct antigenic sites of the cytoplasmic (including PTK) domain of the IR protein (13, 14). Analysis was by SDS-PAGE and fluorography. kd, Kilodalton.

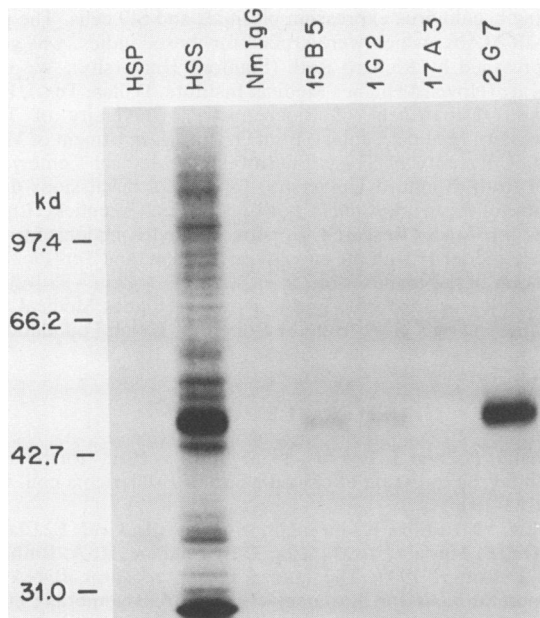


FIG. 4. Phosphorylation of hIR PTK protein in vivo. Sf9 cells were infected with recombinant AchIRPTK 75.5 virus, metabolically labeled after 48 h with $^{32}\text{P}_i$, and fractionated into high-speed pellet (HSP; 1% of total) and supernatant (HSS; 1% of total). Immunoprecipitates were prepared from high-speed supernatant with control normal mouse IgG (NmIgG) and MAb 15B5, 1G2, 17A3, or 2G7. Analysis was by SDS-PAGE and autoradiography. kd, Kilodalton.

plasm of infected cells. To ascertain whether this ~48-kDa protein was indeed soluble, Sf9 cells infected with AchIRPTK 75.5 were metabolically labeled at 48 h postinfection as described above, swollen in hypotonic buffer (10 mM Tris hydrochloride [pH 7.3], 10 mM NaCl, 1.5 mM MgCl_2) for 40 min at 4°C (12), and homogenized (Teflon [E. I. du Pont de Nemours & Co., Inc.] glass), and the distribution of the ~48-kDa band in soluble and particulate fractions after ultracentrifugation at $100,000 \times g_{\text{max}}$ was examined (Fig. 3). The ~48-kDa protein was clearly visible as a major labeled polypeptide in total cell homogenates (Fig. 3, lane 1) and preferentially fractionated into a high-speed supernatant fraction (Fig. 3, cf. lanes 2 and 3). Further, this band was specifically immunoprecipitated by the panel of IR MAbs (lanes 5 to 8), but not by control normal mouse IgG (lane 4). Thus, this recombinant AchIRPTK 75.5 virus directed the synthesis of a soluble hIR cytoplasmic domain in infected Sf9 cells.

hIR PTK domain expressed in Sf9 cells as an active PTK in vivo. What is the state of phosphorylation of the AchIRPTK molecule in the cytoplasm of AchIRPTK 75.5-infected Sf9 cells? When such cells were labeled for 3 h with $^{32}\text{P}_i$ at 48 h postinfection and total cell lysates were fractionated and examined by SDS-PAGE and autoradiography, an ~48-kDa phosphoprotein constituted a major fraction of the labeled cellular phosphoproteins in the high-speed supernatant (Fig. 4, cf. lanes 1 and 2). This labeled protein was not observed in comparable experiments with either mock-infected Sf9 cells or Sf9 cells infected with wild-type AcMNPV (data not shown). That this band was indeed AchIRPTK was demonstrated by the immunoprecipitation of the ~48-kDa protein by three IR MAbs (Fig. 4, lanes 4, 5, and 7), but not by control normal mouse IgG (Fig. 4, lane 3). Note that this labeled protein was also not recognized by MAb 17A3 (Fig.

4, lane 6), whereas MAb 17A3 did recognize the protein labeled with L- ^{35}S methionine and L- ^{35}S cysteine (Fig. 2, lane 6, and Fig. 3, lane 7). This result is consistent with those of previous studies of MAb 17A3, which showed that MAb 17A3 blocks autophosphorylation of the IR, does not recognize the autophosphorylated form of the IR, and (as do all of these MAbs) requires the native conformation of the IR cytoplasmic domain for recognition (13, 14). These results thus suggest that a functional cytoplasmic PTK domain of the IR was expressed in Sf9 cells infected with recombinant AchIRPTK 75.5 virus and that the protein was present in both autophosphorylated and unphosphorylated forms.

The wild-type IR is phosphorylated in an insulin-dependent manner in intact mammalian cells on both tyrosine (autophosphorylation) and serine (by an as yet unidentified non-IR but insulin-dependent serine kinase) residues (9, 15). Excision of the AchIRPTK band phosphorylated in vivo followed by acid hydrolysis and phosphoamino acid analysis demonstrated the incorporation of ^{32}P on both tyrosine and serine residues in such cells (Fig. 5), suggesting that AchIRPTK is likely to be a substrate for an endogenous serine kinase in insect (Sf9) cells.

Affinity-purified AchIRPTK as an active PTK in vitro. To determine whether the hIR PTK expressed in Sf9 cells (designated AchIRPTK) exhibits protein kinase activity in vitro, the protein was immunoprecipitated with MAb 2G7 from lysates of Sf9 cells at 48 h postinfection with AchIRPTK 75.5. When such immune complexes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and divalent cations and then examined by SDS-PAGE and autoradiography, a single ~48-kDa band was observed (Fig. 6A, lane 2), which was not visible in control immune complexes (Fig. 6A, lane 1). Excision of the labeled band followed by acid hydrolysis and phosphoamino acid analysis demonstrated the incorporation of ^{32}P exclusively onto tyrosine residues (Fig. 6B). Furthermore, inclusion of either the synthetic polymer poly(Glu-Tyr) or histone H2b in such phosphorylation reactions resulted in the incorporation of ^{32}P into these exogenous substrates as well. In a typical experiment with such immune complexes, autophosphorylation of AchIRPTK for 30 min in vitro in the

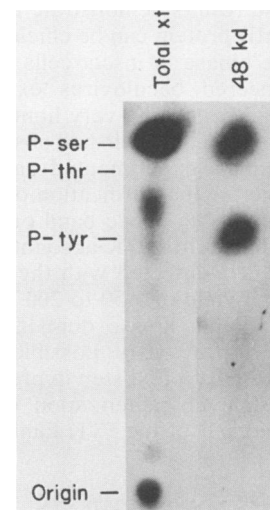


FIG. 5. Phosphoamino acid analysis of total cell extract of Sf9 cells metabolically labeled with $^{32}\text{P}_i$ (Total xt) and gel-purified ~48-kDa AchIRPTK band from the same extract. Positions of standards are indicated: P-ser, phosphoserine; P-thr, phosphothreonine; and P-tyr, phosphotyrosine.

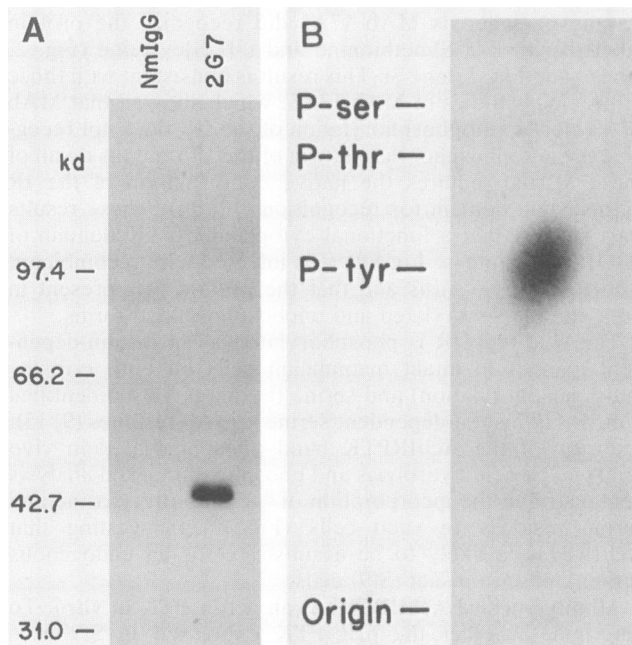


FIG. 6. (A) Autophosphorylation of hIR PTK expressed in AchIRPTK 75.5-infected Sf9 cells in an immune complex assay with MAb 2G7 in vitro. Analysis was by SDS-PAGE and autoradiography. NmIgG, Immunoprecipitation with control normal mouse IgG; 2G7, immunoprecipitation with MAb 2G7. (B) Phosphoamino acid analysis of gel-purified AchIRPTK after autophosphorylation in an immune complex assay with MAb 2G7 in vitro. Positions of standards are indicated: P-ser, phosphoserine; P-thr, phosphothreonine; and P-tyr, phosphotyrosine. kd, Kilodalton.

presence of 100 μ M [γ - 32 P]ATP (specific activity, 11,744 cpm/pmol) resulted in the incorporation of $8,090 \pm 500$ cpm ($n = 3$) into the ~ 48 -kDa AchIRPTK and $480,210 \pm 27,290$ cpm ($n = 3$) into poly(Glu-Tyr). These results demonstrate that AchIRPTK is an active tyrosine-specific kinase in vitro.

In summary, these results demonstrate that the cytoplasmic domain of the hIR protein can be efficiently synthesized as an active soluble kinase in insect cells by the use of an appropriately engineered baculovirus expression vector. This is in striking contrast to the very limited production of inactive kinase in bacteria (unpublished observations). From the results of subsequent experiments from which we have established a scheme for the purification of the AchIRPTK to apparent homogeneity (a single band on a silver-stained gel), we estimated that AchIRPTK accounts for $\sim 5\%$ of the total cell protein in cells infected with the AchIRPTK 75.5 virus, with an overall yield of ~ 50 to $200 \mu\text{g}/10^7$ cells. The generation of this soluble kinase domain in substantially larger quantity than previously possible in mammalian (CHO) cells (5) represents a first step in producing sufficient protein for biophysical characterization of the structural properties of this member of the PTK family.

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