tor and sequenced. The four cDNA inserts (C7, 8, 12, and 14) are 164, 1395, 1014, and 899 bp long, respectively. Two clones (C8 and C12) were then used to make a composite cDNA (2031 bp) and cloned into pBluescript KS vector. This composite cDNA insert covering the entire coding region of *Syp* was sequenced again on both DNA strands.
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19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Activation of a Phosphotyrosine Phosphatase by Tyrosine Phosphorylation

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Regulation of cell proliferation, differentiation, and metabolic homeostasis is associated with the phosphorylation and dephosphorylation of specific tyrosine residues of key regulatory proteins. The phosphotyrosine phosphatase 1D (PTP 1D) contains two amino terminally located Src homology 2 (SH2) domains and is similar to the *Drosophila corkscrew* gene product, which positively regulates the *torso* tyrosine kinase signal transduction pathway. PTP activity was found to be regulated by physical interaction with a protein tyrosine kinase. PTP 1D did not dephosphorylate receptor tyrosine kinases, despite the fact that it associated with the epidermal growth factor receptor and chimeric receptors containing the extracellular domain of the epidermal growth factor receptor and the cytoplasmic domain of either the HER2-*neu*, *kit*-SCF, or platelet-derived growth factor β (βPDGF) receptors. PTP 1D was phosphorylated on tyrosine in cells overexpressing the βPDGF receptor kinase and this tyrosine phosphorylation correlated with an enhancement of its catalytic activity. Thus, protein tyrosine kinases and phosphatases do not simply oppose each other's action; rather, they may work in concert to maintain a fine balance of effector activation needed for the regulation of cell growth and differentiation.

Protein phosphorylation and dephosphorylation are key events in the pathways that regulate cell growth and differentiation. These processes are triggered by secreted polypeptide growth factors or hormones that activate tyrosine kinases to induce specific cellular responses (*1*). The cellular factors that participate in these signaling pathways include polypeptide substrates that contain the Src-homology regions, SH2 and SH3, either alone or in combination with regions that provide enzymatic activity (*2*). The SH2 domain elements of such substrates bind with high affinity to specific phosphorylated tyrosine residues of activated tyrosine kinases and thereby initiate the subsequent intracellular signal transduction cascade (*1*). For example, phospholipase Cγ interacts through its SH2 domain with the cytoplasmic domain of a receptor tyrosine kinase (RTK) and is activated by subsequent phosphorylation (*3*). Protein tyrosine phosphatases (PTPs) are potential regulators of protein tyrosine kinases (PTKs), but it is not clear how these crucial components of phosphotyrosine signaling cascades are activated (*4*).

The large number of currently known members of the PTP family suggests that there may be specificity in PTP-RTK interactions. Recently, a cDNA encoding a PTP designated PTP 1C was cloned from several sources (*5*, *6*). In addition to a single catalytic domain, the structure of this PTP includes a pair of SH2 regions near the NH_2-terminus, which suggested that PTK activity might be regulated by SH2 domain–mediated interaction of tyrosine-phosphorylated RTKs with a PTP.

We identified a PTP, designated PTP 1D, that contains an SH2 domain and has extensive sequence similarity to PTP 1C (*7*). Overlapping clones from a cDNA library from SK-BR-3 mammary carcinoma cells covered 6.8 kb and included in the 5′ portion an open reading frame of 1779 bp that encoded a protein of 593 amino acids with a calculated molecular size of 68 kD (Fig. 1). The coding sequence was flanked by 129 nucleotides of 5′-untranslated sequence and approximately 4.9 kb of 3′-untranslated sequence. The predicted amino acid sequence contains a PTP catalytic domain at the COOH-terminus and two adjacent SH2 domains that are located in the NH_2-terminal portion. PTP 1D and PTP 1C are very similar in their overall structure, with only minor divergence toward the COOH-terminus. The two proteins exhibit 55% sequence identity and 71% overall similarity.

The recently characterized product of the *Drosophila corkscrew* (*csw*) locus (*8*), a gene that participates in signaling by the tyrosine kinase encoded by the *torso* gene (*9*), is similar to PTP 1C and PTP 1D in the SH2 and PTP domains. Within the sequences that could be aligned, *csw* was 69 and 76% similar to PTP 1C and PTP 1D,

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  1 MTSRRWFHPN ITGVEAENLL LTRGVDGSFL ARPSKSNPGD
 41 FTLSVRRNGA VTHIKIQNTG DYYDLYGGEK FATLAELVQY
 81 YMEHHGQLKE KNGDVIELKY PLNCADPTSE RWFHGHLSGK
121 EAEKLLTEKG KHGSFLVRES QSHPGDFVLS VRTGDDKGES
161 NDGKSKVTHV MIRCQELKYD VGGGERFDSL TDLVEHYKKN
201 PMVETLGTVL QLKQPLNTTR INAAEIESRV RELSKLAETT
241 DKVKQGFWEE FETLQQQECK LLYSRKEGQR QENKNKNRYK
281 NILPFDHTRV VLHDGDPNEP VSDYINANII MPEFETKCNN
321 SKPKKSYIAT QGCLQNTVND FWRMVFQENS RVIVMTTKEV
361 ERGKSKCVKY WPDEYALKEY GVMRVRNVKE SAAHDYTLRE
401 LKLSKVGQGN TERTVWQYHF RTWPDHGVPS DPGGVLDFLE
441 EVHHKQESIM DAGPVVVHCS AGIGRTGTFI VIDILIDIIR
481 EKGVDCDIDV PKTIQMVRSQ RSGMVQTEAQ YRFIYMAVQH
521 YIETLQRRIE EEQKSKRKGH EYTNIKYSLA DQTSGDQSPL
561 PPCTPTPPCA EMREDSARVY ENVGLMQQQK SFR*
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Fig. 1. Amino acid sequence of PTP 1D. The deduced amino acid sequence is shown. SH2 and PTP domains are boxed and the latter is shaded. The nucleotide sequence was determined with the chain termination method (*19*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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respectively (8). All three catalytic domains contain characteristic sequence motifs of PTPs—(D or E)YINA, FWXM, and CSAGXGR, which are separated by spacer regions of variable length. Within the approximately 60–amino acid long region between the SH2 tandem repeat and the PTP domain, the sequence GFWEEFE was identified, which is conserved in all three known SH2-containing PTPs but was not found in any other protein sequence contained in the Swiss Protein data base. This sequence may therefore represent a characteristic structural motif for this class of proteins.

Northern (RNA) blot analysis of mouse tissues revealed high amounts of expression of the 6.8-kb mRNA encoding PTP 1D in brain, heart, and kidney; lower amounts were detected in liver, skeletal muscle, testes, and lung, and very low amounts were present in stomach and spleen (*10*). This distribution contrasts with that of PTP 1C, which appears to be expressed predominantly in hematopoietic cell types (6). However, low amounts of PTP 1C mRNA were detected in seven of nine cell lines derived from human mammary carcinomas that were examined. These cell lines generally expressed higher amounts of PTP 1D mRNA (*10*).

We examined the substrate specificity of PTP 1D and PTP 1C and their association with a panel of RTKs in intact cells. We used a system that allows transient overexpression of multiple transfected genes resulting from efficient transactivation of the cytomegalovirus early promoter by ectopically expressed adenovirus transcription factor E1a (*11*) in the human 293 embryonic kidney cell line. Expression constructs containing PTP cDNA in a vector with a cytomegalovirus promoter were transfected into 293 cells together with expression plasmids for one of seven RTKs. These included the receptors for epidermal growth factor (EGF), insulin, insulin-like growth factor–1, and the α and β forms of platelet-derived growth factor receptor (PDGF-R). In the case of HER2-*neu* and p145$^{c\text{-}kit}$, we used chimeric receptors, HER1-2 and EK-R, in which the respective kinase functions were under the control of an EGF receptor (EGF-R) extracellular domain (*12, 13*) (Fig. 2, A to C).

After transfection with equal amounts of expression plasmid and stimulation with the appropriate ligand, cells were lysed and identical portions were analyzed by electrophoresis and immunoblotting with a monoclonal antibody to phosphotyrosine (Fig. 2, A to C). In these experiments, the apparent absence of an effect of ligand on phosphorylation of the PDGF-R and EGF-R resulted from very high overexpression and constitutive activation of basal activity of these receptors in this system. Dephosphorylation activities of PTP 1D and PTP 1C were different, despite the fact that both enzymes were comparably expressed in large amounts. Expression of PTP 1C led to partial or complete dephosphorylation of EGF-R, HER1-2, overexpressed PDGF-R α and β subunits, and the β subunits and unprocessed precursors of insulin receptor (I-R) and insulin-like growth factor–1 receptor (IGF-1–R). In contrast, PTP 1D had no effect on the phosphorylation state of the RTKs examined, with the exception of the EK-R chimera, for which a reduction of about 40% in the phosphotyrosine signal was reproducibly observed (Fig. 2C).

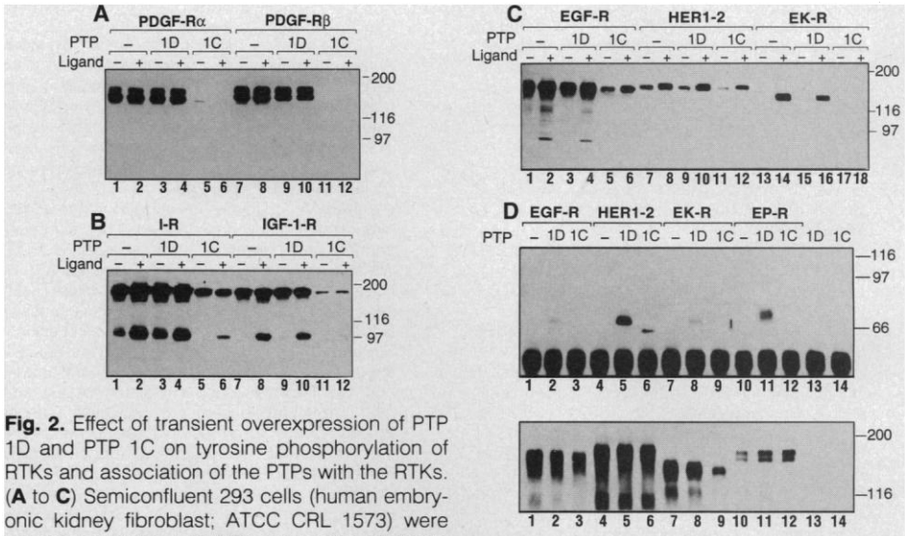

Fig. 2. Effect of transient overexpression of PTP 1D and PTP 1C on tyrosine phosphorylation of RTKs and association of the PTPs with the RTKs. (**A** to **C**) Semiconfluent 293 cells (human embryonic kidney fibroblast; ATCC CRL 1573) were transfected either with RTK expression plasmids alone or with those plasmids and expression vectors encoding human PTP 1D or mouse PTP 1C (*11*). After 24 hours of serum starvation, cells were stimulated for 10 min with the appropriate ligand as indicated. Proteins from portions of cell lysate were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody 5E2 to phosphotyrosine. Blots were developed with an enhanced chemiluminescence (ECL) detection system (Amersham). Molecular size markers are indicated in kilodaltons. Major bands represent PDGF-Rα and -Rβ (A), uncleaved precursors (185 kD) and β subunits (97 kD) of I-R and IGF-1–R (B), and EGF-R, HER1-2 and EK-R proteins (C). Immunoblots were stripped and reprobed with receptor-specific antibodies to confirm that equal amounts of receptor were expressed. Probing with antibodies to PTP 1C and PTP 1D (*20*) revealed comparable ECL signals. (**D**) Association between RTKs and PTPs was determined in 293 cells transfected with expression plasmids coding for chimeric RTKs and PTPs as indicated. After serum starvation, metabolic labeling with [^{35}S]-L-methionine overnight, and stimulation with EGF (50 ng/ml) for 10 min, the EGF-R and the chimeric receptors were immunoprecipitated with the monoclonal antibody 108.1. The precipitates were washed extensively, boiled in SDS sample buffer, and analyzed by SDS-PAGE (7.5% gel). After transfer to nitrocellulose, the blot was probed with a mixture of rabbit antisera to PTP 1D and PTP 1C (*20*) (upper panel). Samples in lanes 13 and 14 were transfected with PTPs alone. Lower panel: Autoradiographic analysis of the precipitated RTKs. Molecular size markers are indicated in kD.

We examined possible association of PTPs with coexpressed RTKs. Our results suggested specific interaction of PTP 1C and PTP 1D with distinct kinase domains. We used three chimeric RTKs with EGF-R extracellular domain fused to the cytoplasmic domains of HER2-*neu* (HER1-2), c-Kit (EK-R), and βPDGF (EP-R) receptors (*14*) and the EGF-R itself (Fig. 2D). After transfection and stimulation with EGF, cells were lysed and proteins from each sample were immunoprecipitated with antibody 108.1 to the extracellular domain of the EGF-R. This strategy eliminated the influence of possible differences in properties of growth factors or antibodies on the experiment and permitted quantitative comparison of the results. Precipitates were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with a mixture of antibodies to PTP 1C and PTP 1D. PTP 1C coimmunoprecipitated with the HER1-2 chimera only (Fig. 2D), whereas PTP 1D associated strongly with HER2-*neu* (HER1-2) and PDGF-Rβ (EP-R) cytoplasmic domains and with lower affinity to EGF-R and c-Kit cytoplasmic domain (EK-R). The PTP 1D band had an apparent molecular size of 68 kD when isolated from cells expressing EGF-R, HER1-2, or EK-R, but it had a larger apparent molecular size when isolated from cells expressing EP-R, suggesting phosphorylation as a result of interaction with the activated PDGF-Rβ cytoplasmic domain.

To examine whether PTP 1D could serve as a substrate for the PDGF-Rβ kinase, 293 cells were transfected with PTP

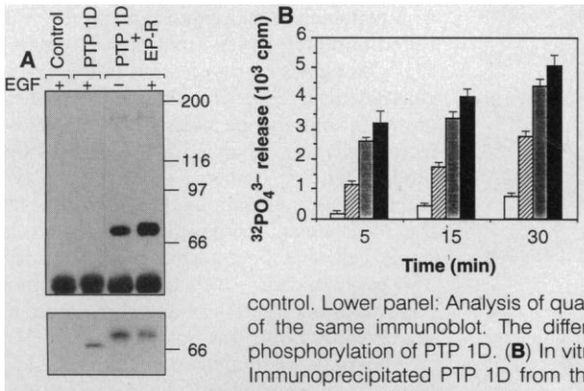

Fig. 3. Phosphorylation and activation of PTP 1D by activated EP-R. (**A**) Immunoprecipitates of PTP 1D from [^{35}S]-L-methionine–labeled 293 cell transfectants expressing PTP 1D either alone or with the EP-R chimera were subjected to immunoblotting with antibodies to phosphotyrosine (upper panel). 293 cells transfected with the vector clone were used as a negative control. Lower panel: Analysis of quantity of PTP 1D by autoradiography of the same immunoblot. The differences in gel mobility are due to phosphorylation of PTP 1D. (**B**) In vitro determination of PTP 1D activity. Immunoprecipitated PTP 1D from the same cell lysates as in (A) was used for an activity assay with the artificial substrate poly(Glu-Tyr) (*15*). Immune complexes bound to Protein A–Sepharose (Pharmacia) were incubated with ^{32}P-labeled substrate (10^4 cpm) for the indicated time at 37°C. After precipitation with 1.5 volumes of trichloroacetic acid (20%), the release of ^{32}P into the supernatant was determined by Cerenkov counting. The data represent means ± SEM from three independent experiments with duplicate time points. Cells were transfected with either control plasmid (open bars), PTP 1D expression vector (striped bars) or PTP 1D and EP-R expression plasmids together with (closed bars) or without (shaded bars) EGF.

1D and EP-R, and proteins from cell lysates were analyzed by immunoprecipitation with antibody to PTP 1D, SDS-PAGE, and immunoblotting with antibody to phosphotyrosine. PTP 1D was phosphorylated on tyrosine in cells transfected with EP-R and this phosphorylation was enhanced by treatment of cells with EGF (Fig. 3A). A small amount of a larger tyrosine phosphorylated protein of about 180 kD was also detected, which is likely to represent the EP-R chimera (*14*).

We examined whether tyrosine phosphorylation by the PDGF-Rβ kinase influenced the catalytic activity of PTP 1D. Identical portions of proteins immunoprecipitated with antibody to PTP 1D from cells overexpressing EP-R and PTP 1D were tested for their ability to release phosphate from [^{32}P]poly(Glu-Tyr) (*15*). Although activity of immunoprecipitated PTP 1D was detectable in the absence of EP-R, immunoprecipitation of PTP 1D from cells also expressing the receptor led to an increase of the activity, which was further enhanced by exposure of cells to the ligand (Fig. 3B). In the presence of equal amounts of PTP 1D and coprecipitated EP-R (see Fig. 3A), the phosphorylation state of PTP 1D correlated with an increase in phosphatase activity. These results demonstrate that regulation of PTP catalytic activity is associated with direct interaction with and tyrosine phosphorylation by a ligand-activated RTK. Because equal amounts of phosphatase and EP-R were present in the immunoprecipitates used in the assay, the increase in phosphatase activity appears to have been caused primarily by phosphorylation of PTP 1D (Fig. 3A).

The recent identification of a large number of tyrosine-specific phosphatases has suggested that these enzymes, which include receptor-like transmembrane, membrane-associated, and cytoplasmic forms, modulate signaling pathways in which proteins are phosphorylated on tyrosine. The target specificity and regulation of PTPs by extracellular ligands or intracellular protein-protein interactions are still poorly understood. The realization that most PTK substrates contain SH2 domains, which mediate interaction with the PTKs, and the discovery of an SH2 domain–containing PTP, PTP 1C, revealed the possibility that SH2 domain–mediated interaction modulates the activity of PTKs. This interpretation was further supported by the finding that the gene product of the *Drosophila corkscrew* locus, which is part of a genetically defined signaling pathway including the RTK encoded by the gene *torso*, the Raf homolog *pole hole*, and the transcription factors *tailless* and *huckebein* (*8*), encodes a PTP containing an SH2 domain.

Whereas PTP 1C displayed promiscuous activity on autophosphorylated receptors, no activity was observed for PTP 1D in the same experiment. Because both PTPs are expressed in several mammary carcinoma cell lines, this suggested that in spite of their close structural similarity the two PTPs have distinct regulatory functions. Alternatively, the apparent lack of substrate specificity by PTP 1C could have been the result of abnormal overexpression, which might cause overactivation and loss of specificity, or it may reflect the requirement for additional cell type–specific factors that are not present in 293 embryonic fibroblasts yet are required for the definition of specificity.

PTP 1D and PTP 1C also differed in their ability to form complexes with RTK cytoplasmic domains. PTP 1C was only coprecipitated with a chimeric EGF-receptor containing the HER2-*neu* cytoplasmic domain, whereas PTP 1D associated with several receptors but did not cause dephosphorylation. Although we cannot be certain of whether our analysis failed to detect minor changes in the phosphorylation state of specific tyrosine residues in the RTKs tested, we interpret our data to suggest that PTP 1D interacts tightly, presumably via its SH2 sequences, with phosphotyrosine residues in the activated PDGF-Rβ cytoplasmic domain, thereby protecting them from dephosphorylation and thus protecting the receptor from inactivation. In turn, PTP 1D is phosphorylated on tyrosine, which activates its catalytic function and may lead to activation of a positive signaling potential by dephosphorylation of phosphotyrosines that negatively regulate the signaling potential of other polypeptides, such as the Src-type tyrosine kinases (*16*).

In conjunction with the genetic evidence for a positive regulatory function for *csw*, our observations suggest a role for PTP 1D in the definition or amplification, rather than negative control, of PDGF-R–mediated signals.

Note added in proof: After submission of this manuscript, isolation of a cDNA clone encoding the same PTP, designated SH-PTP2, was reported (*17*). The PTP Syp (*18*) appears to be a COOH-terminal splice variant (amino acids 549 to 593) of the mouse homolog of PTP 1D. The failure of both groups to demonstrate regulated phosphatase activity is likely due to the fact that Freeman *et al.* (*17*) used a truncated version expressed in *Escherichia coli* lacking all of the NH_2-terminal and most of the COOH-terminal SH2 domains and did not study RTK interaction and phosphorylation, whereas Feng *et al.* (*18*) used different experimental conditions, a different substrate, and an alternatively spliced mouse sequence.

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7. A pool of degenerated primers corresponding to all sequences coding for the conserved PTP motifs (D or E or G)(S or N)(D or N)Y I N A, (sense) and K C (A or D)(Q or E)Y W P, (antisense) was designed for cloning with the polymerase chain

reaction (PCR). As a template, cDNA was generated from polyadenylated RNA (5 μg) of the breast cancer cell line SK-BR-3 with reverse transcriptase from avian myeloblastosis virus (Boehringer-Mannheim). The 220-bp PCR product was subcloned into the Bluescript vector with restriction enzyme (Eco RI and Bam HI) cleavage sequences attached to the polymerase chain reaction primers (Stratagene). One clone (P 158) of the 120 sequenced was identified to represent a different PTP and was therefore used as a probe to screen a SK-BR-3 cell, λZAP cDNA library. Sequencing of the longest clone revealed an open reading frame without an upstream termination signal. Full-length cloning was completed by rescreening of the library and by primer extension with an appropriate 5′ oligonucleotide.

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20. Antisera to PTP 1D and PTP 1C were obtained by immunizing rabbits with fusion proteins consisting of glutathione S-transferase (Pharmacia) and portions of NH_2-terminal sequences of PTP 1D (residues 28 to 219) and PTP 1C (residues 55 to 302), respectively.
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Expression Cloning and Signaling Properties of the Rat Glucagon Receptor

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Glucagon and the glucagon receptor are a primary source of control over blood glucose concentrations and are especially important to studies of diabetes in which the loss of control over blood glucose concentrations clinically defines the disease. A complementary DNA clone for the glucagon receptor was isolated by an expression cloning strategy, and the receptor protein was expressed in several kidney cell lines. The cloned receptor bound glucagon and caused an increase in the intracellular concentration of adenosine 3′, 5′-monophosphate (cAMP). The cloned glucagon receptor also transduced a signal that led to an increased concentration of intracellular calcium. The glucagon receptor is similar to the calcitonin and parathyroid hormone receptors. It can transduce signals leading to the accumulation of two different second messengers, cAMP and calcium.

Glucagon is a 29–amino acid pancreatic hormone that affects the production and distribution of glucose by its target organ, the liver. Glucagon functions to maintain basal concentrations of glucose and is a key hormone in the pathogenesis of diabetes. Glucagon binds to receptors in liver and activates two enzymatic pathways, glycogenolysis and gluconeogenesis, which result in the production of glucose (*1*). It is assumed that these actions of glucagon on the liver result when glucagon binds its hepatic receptor (or receptors) and activates adenylate cyclase, thereby increasing the intracellular concentration of cAMP. However, glucagon may also cause hydrolysis of inositol phospholipids (*2*, *3*) and increases in intracellular calcium concentrations (*4*). Two types of hepatic glucagon receptor have been proposed, and it has been suggested that they signal via two different intracellular messengers (*2*). Despite considerable effort, it has not been possible to isolate a cDNA for the glucagon receptor. We have used an expression cloning strategy to isolate a cDNA that encodes a functional hepatic receptor for glucagon. Like the parathyroid hormone (PTH) and calcitonin (CT) receptors (*5*, *6*), the glucagon receptor, when stimulated, gives rise to increased intracellular concentrations of cAMP and calcium.

We constructed a rat liver cDNA library in a mammalian cell expression vector and tested miniprep DNAs from pools containing 5000 clones by transfection into COS-7 cells (African Green monkey kidney cells) grown on microscope slides (*7*). The transfected cells were analyzed after 72 hours by binding with ^{125}I-labeled glucagon and by emulsion autoradiography. One pool out of the 100 screened reproducibly gave rise to a single cell that bound glucagon per slide. This pool was successively broken down into portions of one-tenth as many clones until a single clone, pLJ4, was isolated.

The pLJ4 plasmid contains a 1.9–kilobase pair insert that encodes a 485–amino acid protein with a predicted molecular size of 54,962 daltons (Fig. 1). The receptor encoded by pLJ4 is related to the CT (*8*), PTH (*5*), secretin (*9*), vasoactive intestinal peptide (VIP) (*10*), and glucagon-like peptide I (GLP I) (*11*) receptors (24, 31, 34, 35, and 42% amino acid identity, respectively). Analysis of hydropathy (*12*) revealed eight clusters of hydrophobic amino acids. The NH_2-terminal cluster does not contain a likely site for signal peptide cleavage (*13*). The remaining hydrophobic clusters correspond to seven transmembrane domains (TMDs), as seen with the other members of the secretin receptor family. Alignment of the rat secretin, rat PTH, porcine CT, and pLJ4 receptor sequences shows that amino acids within and adjacent to the TMDs are conserved (Fig. 1). Just before the sixth TMD the sequence RLAR appears in pLJ4, and a similar sequence motif, KLAK, was shown to be important for the interaction of the β2-adrenergic receptor with the adenylate cyclase–stimulating guanosine triphosphate–binding protein ($G_{s\alpha}$) (*14*). The COOH-terminal segment of pLJ4 shows less similarity to the CT, PTH, and secretin receptors, except for a segment of 16 amino acids adjacent to the seventh TMD. There are four potential N-linked glycosylation sites located in an extended hydrophilic stretch (amino acids 30 to 140) and six cysteine residues conserved among the secretin receptor family in this same segment. This segment may be an extracellular region of the receptor analogous to those predicted for the other receptors in this family.

We expressed pLJ4 transiently in COS-7 cells and established stable baby hamster kidney (BHK) cell lines that express pLJ4. Both types of cells bound ^{125}I-labeled glucagon with similar affinity to that of the receptors in rat liver membranes (*15*). Competition with unlabeled glucagon yielded nearly identical sigmoidal curves for glucagon binding by BHK cell or rat liver membrane preparations (Fig. 2). As indicated by Scatchard analysis, the apparent dissociation constants (K_d's) were 37 nM for the cloned receptor and 38 nM for rat liver membranes. These K_d's correspond closely to published

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