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Cloning and Expression of Chicken Protein Tyrosine Phosphatase Gamma

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Abstract
A 5,403 bp cDNA encoding chicken protein tyrosine phosphatase gamma (PTPγ) was isolated and sequenced. The predicted open reading frame of 1,422 amino acids (aa) includes 742 aa of extracellular (EC) domain, 26 aa of transmembrane (TM) domain and 634 aa of intracellular domain. The chicken PTPγ has a 86.7% aa identity to its human homolog and contains the carbonic anhydrase-like domain and fibronectin type III homologous regions in the EC domain, as well as the tandem linked catalytic sequences in the cytoplasmic domain. However, the chicken PTPγ lacks 29 aa immediate downstream of the putative TM domain in comparison with its human counterpart. Northern analysis revealed the presence of two transcripts of 6.3 and 9.5 kb in various tissues. The cytoplasmic domain of the PTPγ could be expressed as an enzymatically active form in SF9 insect cells. PTPγ could also be expressed in normal and *src*-transformed NIH3T3 and Rat1 cells as a gag-PTP fusion protein, but no detectable effects on growth and colony formation of these cells were observed.

Tyrosine phosphorylation is one of the most important protein modifications involved in modulating a number of physiological and pathological processes, including cell growth, differentiation, and neoplastic transformation [24]. The state of such phosphorylation depends on the relative activities of two families of enzymes with opposing activities, namely protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).

PTPs can be divided into membrane-spanning receptor-like proteins (RPTP) and intracellular PTPs which can be further grouped based on the presence of SH2 domains [10, 13] RPTPs include RPTP α, β, γ, δ, ε, λ, μ, CD45, LAR, and DEP-1. All of them have an extracellular (EC) domain, a transmembrane (TM) domain and a cytoplasmic domain featuring two tandem catalytic sequences. Studies on the two tandem catalytic domains suggest that the N-terminal catalytic domain is more active than the C-terminal one, which may also have a regulatory function. A study of substrate interaction with PTPs has suggested that chemical features of the primary sequence surrounding the dephosphorylation site of the substrate contribute to its recognition by the enzymes [42]. Very little is known about the physiological substrates for PTPs in general. However, MAP kinase was shown to be a substrate of MKP-1, a dual specificity phosphatase [36] and c-src was shown to be dephosphorylated by PTPα [44]. In addition, when different PTPs were coexpressed with a panel of receptor PTKs (RPTK), clear differences were observed in susceptibility of different RPTKs to dephosphorylation by PTP1B, T-cell phospha-

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tase (TC-PTP), and CD45 [20]. Aside from differential substrate recognition, this observation could also imply that cellular compartmentalization of a PTP may play an important role in its substrate interaction and biological function.

It is generally assumed that PTPs serve to negatively regulate PTKs and thus cell growth. Emerging evidence, however, suggests that the effect of PTP on cell growth can be either negative or positive. Based on the observation that RPTPγ maps to a region of human chromosome 3, 3p21, frequently deleted in renal and lung carcinomas, RPTPγ has been considered a candidate of tumor suppressor genes [19]. Another RPTP that is implicated to have a negative effect on cell growth is DEP-1 [27]. It was reported that the expression level of DEP-1 increased gradually with increasing cell contact and was initiated before saturation cell density was reached. This observation suggests that DEP-1 may be involved in contact inhibition of cell growth. PTP1C was also shown to be predominantly a negative regulator of growth factor signaling in hematopoietic cells [17]. By contrast, RPTPα may positively regulate cell growth. Evidence in support of this includes that overexpression of RPTPα in rat embryo fibroblasts leads to cell transformation [44] probably through activation of signaling proteins, including Src, MAPK, and Jun [43]. The negative regulatory site of *src*, Tyr 527, was preferentially dephosphorylated, and MAPK and Jun were activated and translocated to the nucleus in RPTPα transfected cells [43]. Other studies suggested that Grb2 could bind to RPTPα and the binding site was mapped to Tyr 789 both in vitro and in vivo [8, 10, 34]. Syp, a widely expressed SH2-containing PTP, has been shown to participate in signal transduction leading to mitogenesis [3, 32, 37, 39]. Syp has been shown to interact with a number RPTKs through its SH2 domain resulting in enhancement of its enzymatic activity [39]. Thus, PTP and PTK, having opposite enzymatic activities, could function in concert or antagonistically in regulation of cell growth and differentiation.

RPTPβ (also known as PTPζ), which is closely related to RPTPγ, has been cloned and shown to be brain specific [18, 21, 30]. RPTPβ was shown to be expressed in the form of proteoglycan and binds to the extracellular matrix protein tenascin [1]. The EC domains of receptor PTPs have diverse structural features [26]. Interestingly, the EC domains of RPTPγ and β contain stretches of amino acids with striking homology to the carbonic anhydrase (CAH) domain and fibronectin III repeats [2, 18, 21, 30]. The CAH domain of RPTPβ was shown to be a functional ligand for the axonal cell recognition molecule contactin [28]. Those interactions were suggested to have some role in signalling for neural development. It is interesting to note that murine L cells have lost one *ptpγ* allele and sustained an intragenic deletion within the CAH domain in the other. Whether there exist bona fide ligands for RPTPs similar to those for RPTKs remains an open question. However, aside from the presumed interaction with ligands, the EC domains of certain RPTKs, such as RPTPμ, were shown to be able to bind to each other homophilically suggesting a role in cell-cell interaction [4].

In this paper, we present the cloning of chicken PTPγ and study of its biochemical and biological properties.

Materials and Methods

Cells and Colony Formation Assay

NIH3T3 and Rat1 cell lines transformed by *src* were isolated by infection with subgroup A Rous sarcoma virus [L.-H. Wang, unpubl. result]. Colony formation in soft agar was done as described previously [12].

Library Screening

The original chicken PTPγ 4.1 clone was isolated by low stringency screening of a λgt10 chicken kidney cDNA library [7] with a probe of full-length human CD45 cDNA [33]. Subsequently, a 5′ fragment released from the 4.1 clone by digestion with EcoR1 (the 5′ cloning site) and Bsa1 was used as a probe to screen a λgt10 chicken brain cDNA library [22] under high stringency condition. Several upstream cDNA clones were isolated. Then the further 5′ fragment was used as a probe to screen the library again. After several rounds of screening, a set of 5′ cDNA clones were isolated which, together with the 4.1 cDNA clone, allowed us to assemble into a composite cDNA of 5,403 bp. Hybridization was carried out at 42 °C overnight in a solution containing 5 × SSC, 50% formamide, 20 m*M* Tris-HCl (pH 7.4), 0.5% SDS, 1 m*M* EDTA, 4 mg/ml salmon sperm DNA and 10^7 cpm/ml of probe. For low stringency condition, filters were washed at 37 °C for 30 min in 2 × SSC, 0.2% SDS and then processed for autoradiography. For high stringency condition, filters were washed in 0.1 × SSC, 0.2% SDS at 65 °C for 30 min.

RNA Analysis

Total RNAs from various chicken tissues were isolated by guanidine thiocyanate extraction followed by centrifugation on a cesium chloride cushion according to the published methods [11]. Polyadenylated RNAs were purified as described previously [40]. For Northern blotting analysis, RNA electrophoresis and transfer to nylon membrane were done as described [31]. Riboprobe was prepared from the clone 4.1 subcloned into the pBluescript vector (Strategene) using T7 RNA polymerase. Hybridization was carried out in a solution containing 5 × SSC, 1% SDS, 50 m*M* Na_3PO_4 (pH 6.5), 8 × Denhart's solution, 0.5 mg/ml yeast RNA, 5 μg/ml poly(C) at 72 °C overnight. Nylon membranes were washed 3 times in 0.1 × SSC, 0.2% SDS at 78 °C.

Protein Analysis

Metabolic labeling, protein extraction, immunoprecipitation and gel electrophoresis were carried out as described previously [14, 23, 45, 46].

Generation of Antibody

Clone 4.1 was inserted into the expression vector pSEM3 at SmaI and EcoR1 sites to generate a lacZ-PTP fusion cDNA. The 160-kD fusion protein was expressed in bacteria strain W3110, extracted from the insoluble fraction and purified by gel electrophoresis. The protein band was excised from the gel and eluted out. The immunization of rabbit and collection of serum were done following standard procedures.

Expression of the Cytoplasmic Domain of PTPγ in SF9 Cells Using Baculovirus Vector

To generate the initiation codon for the PTP cDNA, the authentic initiation codon of polyhedron that had been mutated to ACT in the PVL 1393 vector was mutated back to ATG by polymerase chain reaction (PCR) using the following primers: 5′ primer, 5′ATTATAGTTGCTGATATCAT (the EcoRV site used for cloning is underlined), and 3′ primer, 5′CCCGGGATCCGACGCCCGATGGTGGGACGGTATGAATAATCCGG**CA**TATT (the BamH1 cloning site is underlined. The initiation codon is in bold face and the mutation nucleotides A and C are italicized). The 3′ primer (– strand) corresponds to a sequence from +46 to –3 of PVL 1393 vector, but has a T to G change at position +3 and an A insertion at position +36 to generate an initiation codon and reading frame-shift respectively (fig. 5A). The PCR product was digested with EcoRV and BamH1 and ligated to PVL 1393 which was previously cut by the same enzymes. The resulting plasmid which has an authentic polyhedron initiation codon and a changed reading frame starting at codon 12 was named PVL1394. The cytoplasmic cDNA fragment corresponding to amino acid (aa) residues 756–1,422 of PTPγ was released from the clone 4.1 in pBluescript vector by digestion with BstU1 and EcoR1 and inserted into the PVL1394 vector at SmaI and EcoR1 sites (fig. 5). As a result, 12 aa from the baculoviral polyhedron and 3 aa from the linker sequence were fused to the cytoplasmic portion of PTPγ. The expressing plasmid was cotransfected into SF9 cells with the linearized viral helper DNA (Pharmingen). Recombinant virus seletion, infection and maintenance of SF9 culture were carrried out according to the described procedures [35].

Construction of the gag-PTP Fusion Protein Expression Plasmid and Expression of the Fusion Protein in Mammalian Cells

A XbaI-EcoR1 cDNA fragment of PTPγ including the TM and entire cytoplasmic domain was cloned into the pBluescript vector. Then a XbaI fragment released from that vector, including the entire PTP insert and linker sequences, was transferred to the pECE vector [9] to give rise to pECE-PTP plasmid. A SacI-EcoRV DNA fragment of pUR2H1 [46] including the 5′ noncoding and first 150 aa of *gag* p19 was ligated to the pECE-PTP plasmid at the SacI site and XbaI site (which was generated by partial digestion and then blunted). Transfection was done as previously described [46].

Assay of PTP Activity

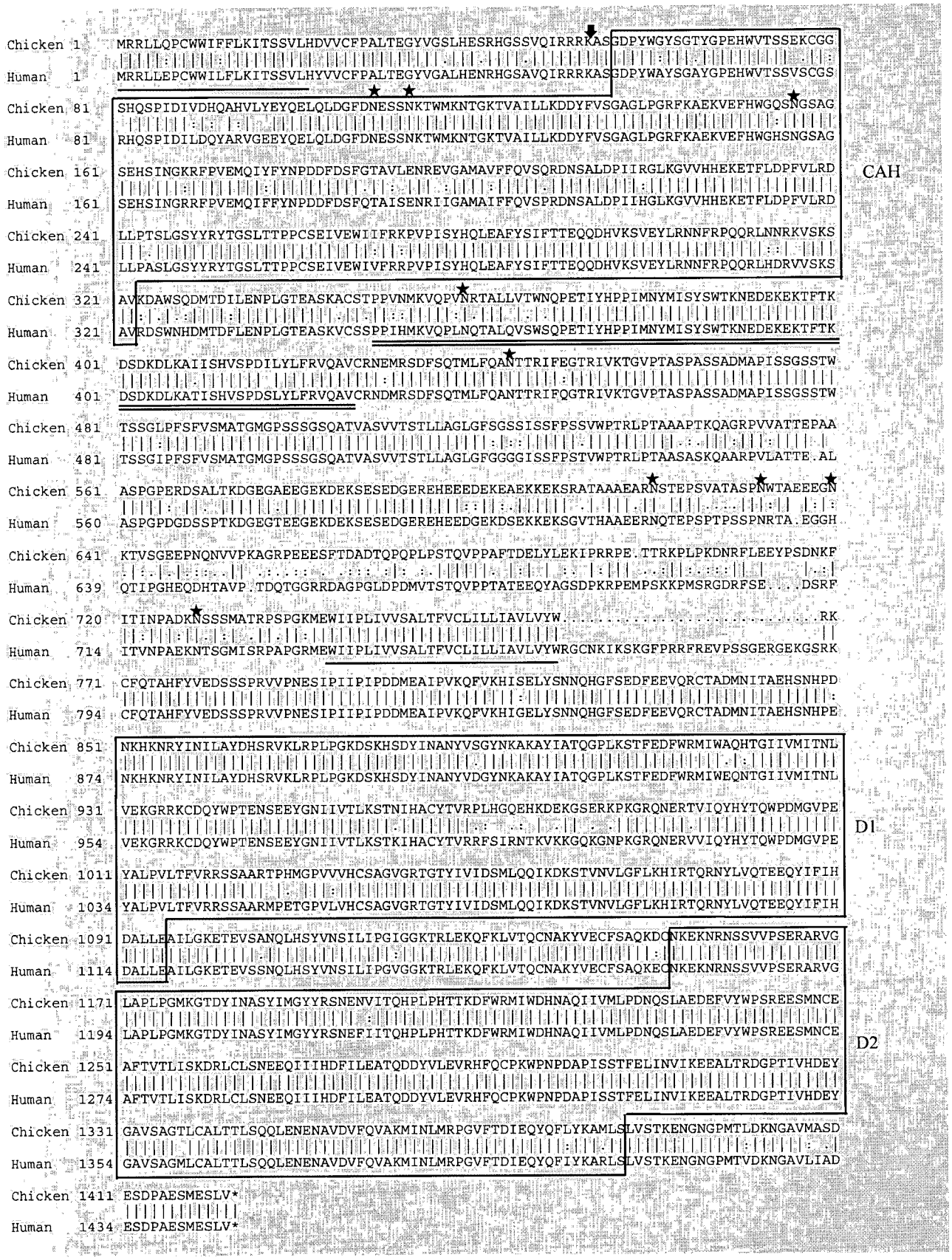
The peptide substrate Raytide (Oncogene Science) was tyrosine phosphorylated with $pp60^{src}$ immunoprecipitated from an extract of Rous sarcoma virus-infected CEF. The kinase reaction was carried out according to the company provided protocol (Oncogene Science). After removing the protein A-bound Src immunocomplex, the peptide in the supernatant (50 µl) was precipitated by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid (TCA) in 20 m*M* Na_3PO_4, 0.1 ml 5 mg/ml BSA and the mixture was incubated on ice for 10 min. After brief spin at 12,000 rpm, the precipitates were washed with 20% TCA in 20 m*M* Na_3PO_4 and then dissolved in 0.2 *M* Tris-HCl (pH 8.0). Phosphatase assays were carried out by adding the labeled Raytide ($1–5 \times 10^5$ cpm) to PTP immunoprecipitates resuspended in 50 µl reaction buffer containing 25 m*M* HEPES (pH 7.3), 5 m*M* EDTA, and 10 m*M* DTT. The reaction was carried out for 15 min at 37 °C and then terminated by adding 20% TCA to a final concentration of 5%. After removing the PTP immunoprecipitates, the supernatant-containing phosphate (Pi) and Raytide was spotted on a sheet of Whatman paper and developed descendingly with 5% TCA. The amount of Pi released was counted by scintillation counter.

Results and Discussion

Isolation and Sequencing of Chicken PTPγ cDNAs

Taking advantage of the conservation of catalytic domains among different PTPs, a chicken kidney cDNA library was screened with a full-length CD45 cDNA probe under a low stringency condition. A 4.1-kb cDNA clone was isolated. Subsequently a chicken brain cDNA library was screened using a 5′ cDNA probe derived from the original cDNA clone under high stringency. After several rounds of screening, a set of cDNA clones with overlapping sequences were isolated and subcloned into the pBluescript vector and sequenced on both strands. Those overlapping sequences were assembled into a total cDNA length of 5,403 bp (the sequence has been deposited in Genbank, the accession number is U38349). This 5.4-kb cDNA contains an open reading frame of 1,422 aa which has two hydrophobic aa stretches. One is at the very amino-terminal end and is presumably the signal peptide, the other is at aa 743–768 which is most likely the TM domain. Sequence comparison between human PTPγ [2, 16] and our cDNA showed that they share 86.7% identity at the aa level and 82% identity at the nucleotide level. Thus we think this cDNA represents the chicken PTPγ.

Fig. 1. Comparison of predicted aa sequence of chicken PTPγ with that of the human homolog. Symbols: |, identical aa: and., similar aa. CAH-like domain and phosphatase domains 1 (D1) and 2 (D2) are boxed. The hydrophobic sequences are underlined. Fibronectin III repeat is double-underlined. The asterisks and the arrow represent glycosylation sites and signal peptide cleavage site, respectively.


Chicken 1 MRRLLQPCWWIFFLKITSSVLHDVVCFPALTEGYVGSLHESRHGSSVQIRRRKASGDPYWGYSGTYGPEHWVTSSEKCGG
Human 1 MRRLLEPCWWILFLKITSSVLHYVVCFPALTEGYVGALHENRHGSAVQIRRRKASGDPYWAYSGAYGPEHWVTSSVSCGS
Chicken 81 SHQSPIDIVDHQAHVLYEYQELQLDGFDNESSNKTWMKNTGKTVAILLKDDYFVSGAGLPGRFKAEKVEFHWGQSNGSAG
Human 81 RHQSPIDILDQYARVGEEYQELQLDGFDNESSNKTWMKNTGKTVAILLKDDYFVSGAGLPGRFKAEKVEFHWGHSNGSAG
Chicken 161 SEHSINGKRFPVEMQIYFYNPDDFDSFGTAVLENREVGAMAVFFQVSQRDNSALDPIIRGLKGVVHHEKETFLDPFVLRD
Human 161 SEHSINGRRFPVEMQIFFYNPDDFDSFQTAISENRIIGAMAIFFQVSPRDNSALDPIIHGLKGVVHHEKETFLDPFVLRD
Chicken 241 LLPTSLGSYYRYTGSLTTPPCSEIVEWIIFRKPVPISYHQLEAFYSIFTTEQQDHVKSVEYLRNNFRPQQRLNNRKVSKS
Human 241 LLPASLGSYYRYTGSLTTPPCSEIVEWIVFRRPVPISYHQLEAFYSIFTTEQQDHVKSVEYLRNNFRPQQRLHDRVVSKS
Chicken 321 AVKDAWSQDMTDILENPLGTEASKACSTPPVNMKVQPVNRTALLVTWNQPETIYHPPIMNYMISYSWTKNEDEKEKTFTK
Human 321 AVRDSWNHDMTDFLENPLGTEASKVCSSPPIHMKVQPLNQTALQVSWSQPETIYHPPIMNYMISYSWTKNEDEKEKTFTK
Chicken 401 DSDKDLKAIISHVSPDILYLFRVQAVCRNEMRSDFSQTMLFQANTTRIFEGTRIVKTGVPTASPASSADMAPISSGSSTW
Human 401 DSDKDLKATISHVSPDSLYLFRVQAVCRNDMRSDFSQTMLFQANTTRIFQGTRIVKTGVPTASPASSADMAPISSGSSTW
Chicken 481 TSSGLPFSFVSMATGMGPSSSGSQATVASVVTSTLLAGLGFSGSSISSFPSSVWPTRLPTAAAPTKQAGRPVVATTEPAA
Human 481 TSSGIPFSFVSMATGMGPSSSGSQATVASVVTSTLLAGLGFGGGGISSFPSTVWPTRLPTAASASKQAARPVLATTE.AL
Chicken 561 ASPGPERDSALTKDGEGAEEGEKDEKSESEDGEREHEEEDEKEAEKKEKSRATAAAEARNSTEPSVATASPNWTAEEEGN
Human 560 ASPGPDGDSSPTKDGEGTEEGEKDEKSESEDGEREHEEDGEKDSEKKEKSGVTHAAEERNQTEPSPTPSSPNRTA.EGGH
Chicken 641 KTVSGEEPNQNVVPKAGRPEEESFTDADTQPQPLPSTQVPPAFTDELYLEKIPRRPE.TTRKPLPKDNRFLEEYPSDNKF
Human 639 QTIPGHEQDHTAVP.TDQTGGRRDAGPGLDPDMVTSTQVPPTATEEQYAGSDPKRPEMPSKKPMSRGDRFSE...DSRF
Chicken 720 ITINPADKNSSSMATRPSPGKMEWIIPLIVVSALTFVCLILLIAVLVYW.....RK
Human 714 ITVNPAEKNTSGMISRPAPGRMEWIIPLIVVSALTFVCLILLIAVLVYWRGCNKIKSKGFPRRFREVPSSGERGEKGSRK
Chicken 771 CFQTAHFYVEDSSSPRVVPNESIPIIPIPDDMEAIPVKQFVKHISELYSNNQHGFSEDFEEVQRCTADMNITAEHSNHPD
Human 794 CFQTAHFYVEDSSSPRVVPNESIPIIPIPDDMEAIPVKQFVKHIGELYSNNQHGFSEDFEEVQRCTADMNITAEHSNHPE
Chicken 851 NKHKNRYINILAYDHSRVKLRPLPGKDSKHSDYINANYVSGYNKAKAYIATQGPLKSTFEDFWRMIWAQHTGIIVMITNL
Human 874 NKHKNRYINILAYDHSRVKLRPLPGKDSKHSDYINANYVDGYNKAKAYIATQGPLKSTFEDFWRMIWEQNTGIIVMITNL
Chicken 931 VEKGRRKCDQYWPTENSEEYGNIIVTLKSTNIHACYTVRPLHGQEHKDEKGSERKPKGRQNERTVIQYHYTQWPDMGVPE
Human 954 VEKGRRKCDQYWPTENSEEYGNIIVTLKSTKIHACYTVRRFSIRNTKVKKGQKGNPKGRQNERVVIQYHYTQWPDMGVPE
Chicken 1011 YALPVLTFVRRSSAARTPHMGPVVVHCSAGVGRTGTYIVIDSMLQQIKDKSTVNVLGFLKHIRTQRNYLVQTEEQYIFIH
Human 1034 YALPVLTFVRRSSAARMPETGPVLVHCSAGVGRTGTYIVIDSMLQQIKDKSTVNVLGFLKHIRTQRNYLVQTEEQYIFIH
Chicken 1091 DALLEAILGKETEVSANQLHSYVNSILIPGIGGKTRLEKQFKLVTQCNAKYVECFSAQKDCNKEKNRNSSVVPSERARVG
Human 1114 DALLEAILGKETEVSSNQLHSYVNSILIPGVGGKTRLEKQFKLVTQCNAKYVECFSAQKECNKEKNRNSSVVPSERARVG
Chicken 1171 LAPLPGMKGTDYINASYIMGYYRSNENVITQHPLPHTTKDFWRMIWDHNAQIIVMLPDNQSLAEDEFVYWPSREESMNCE
Human 1194 LAPLPGMKGTDYINASYIMGYYRSNEFIITQHPLPHTTKDFWRMIWDHNAQIIVMLPDNQSLAEDEFVYWPSREESMNCE
Chicken 1251 AFTVTLISKDRLCLSNEEQIIIHDFILEATQDDYVLEVRHFQCPKWPNPDAPISSTFELINVIKEEALTRDGPTIVHDEY
Human 1274 AFTVTLISKDRLCLSNEEQIIIHDFILEATQDDYVLEVRHFQCPKWPNPDAPISSTFELINVIKEEALTRDGPTIVHDEY
Chicken 1331 GAVSAGTLCALTTLSQQLENENAVDVFQVAKMINLMRPGVFTDIEQYQFLYKAMLSLVSTKENGNGPMTLDKNGAVMASD
Human 1354 GAVSAGMLCALTTLSQQLENENAVDVFQVAKMINLMRPGVFTDIEQYQFIYKARLSLVSTKENGNGPMTVDKNGAVLIAD
Chicken 1411 ESDPAESMESLV*
Human 1434 ESDPAESMESLV*
CAH
D1
D2

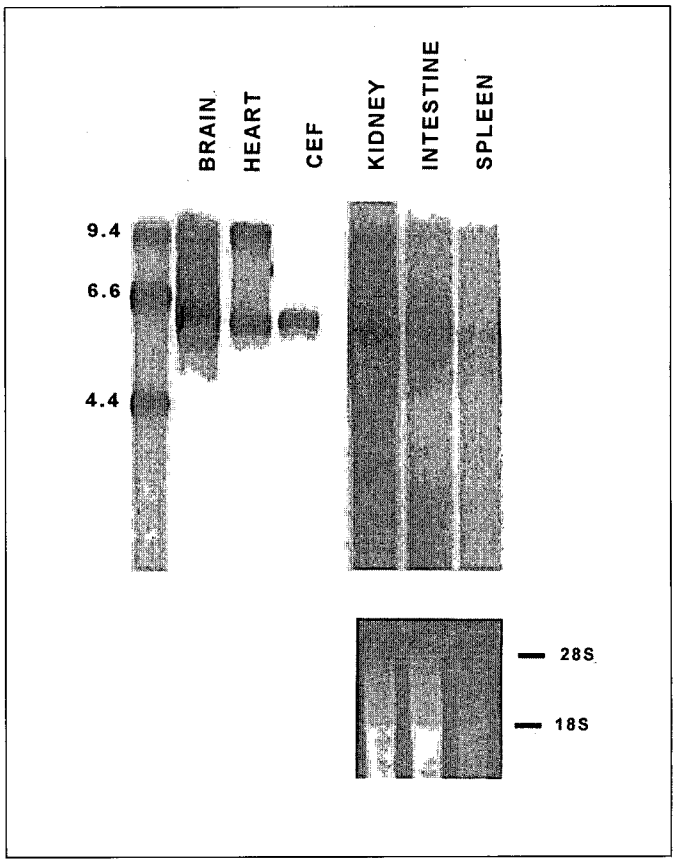


Fig. 2. Northern analysis of chicken PTPγ mRNA in different tissues. 10 μg of poly(A)-containing RNA was used in each sample. Riboprobe transcribed from clone 4.1 (fig. 1) in pBluescript vector was used for hybridization. Molecular weight markers are in kb. RNA samples were separated on an agarose gel, some of them were also stained with ethidium bromide and photographed as shown under the Northern blot.

Sequences of human and mouse PTPγ revealed that their EC domains contain a region of 266 aa residues with striking sequence similarity to the enzyme CAH and a sequence resembling fibronectin type III repeat. The cytoplasmic domain of PTPγ contains two tandem stretches of typical PTP catalytic sequences [1]. However, the second catalytic domain has an Asp residue at position 1351 instead of a conserved Cys residue thought to be essential for the catalytic activity. Both chicken and human PTPζ also lack the conserved cysteine residue [18, 30]. All those domains and structural features are well conserved in chicken RPTPγ (fig. 1). However, the region encompassing about 100 aa residues immediate upstream of the TM domain is relatively divergent among the three species. Interestingly, a 29 aa stretch immediately carboxyl to the TM domain is deleted in the chicken PTPγ in comparison with the human and mouse homologs. We have isolated several independent cDNA clones spanning this region. They all have this 29 aa deletion, suggesting that this is a genuine difference among chicken and mammalian species. Chicken PTPγ has nine potential N-glycosylation sites. Eight of them are conserved in human whereas six of them are conserved in murine homolog.

Expression of Chicken PTPγ mRNA in Different Tissues

Two transcripts of 6.3 and 9.5 kb were detected at varying ratios in different tissues by Northern blot analysis (fig. 2). They are bigger than the murine counterparts which were reported to be 5.5 and 8.5 kb [1]. Only the 6.3-kb transcript was detected in CEF. RNA slot-blot hybridization revealed that PTPγ mRNA was detected in various chicken tissues including brain, heart, intestine, lung, kidney, ovary, spleen, and stomach, but not in bursa, liver, muscle, and thymus (data not shown). The cell-type-specific differences in the size of the PTPγ mRNAs could have resulted from alternative splicing. Alternatively, they may represent closely related genes. The expression profile of PTPγ is in contrast to that of RPTPβ (PTPζ) which is neural specific [6, 21, 30]. This implies distinct functions for these two otherwise structurally related RPTPs.

Expression of the Cytoplasmic Domain of Chicken PTPγ in SF9 Cells

To study biochemical properties of the chicken PTPγ, its cytoplasmic domain was engineered into a modified PVL1393 vector that contains a baculovirus polyhedron promoter and initiation codon (fig. 3A). The expression plasmid was cotransfected into SF9 cells with the linearized helper viral genomic DNA containing a lethal deletion so that only the recombinant virus generated by recombination between the helper DNA and the expression plasmid can survive. After transfection, the recombinant virus was harvested and the titer was determined to be 10^6 PFU/ml. The PTPγ protein expression was detected by metabolic labelling of the recombinant virus-infected cells. The result showed that a protein of expected size was detected in the recombinant virus-infected cells at a very low level, but not in the wild-type virus-infected cells (fig. 3B). Phosphatase assays showed that the cytoplasmic domain was enzymatically active (fig. 4, lanes 5 and 6). The protein was initially detected at 24 h after infection and reached the highest level at 48 h and decreased thereafter (fig. 3B).

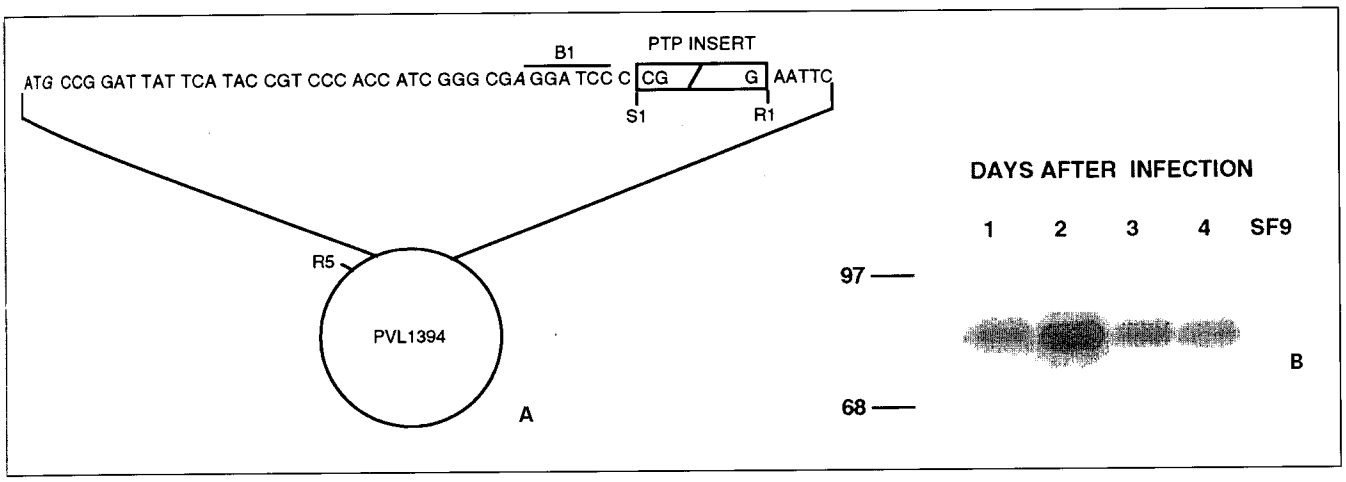


Fig. 3. Expression of the cytoplasmic domain of PTPγ in SF9 cells. **A** Schematic representation of the expression plasmid. The pVL 1394 vector is derived from pVL1393 with substitution of T by G at position +3 and an A insertion at position +36 indicated as italics. R5, EcoR5; B1, BamH1; S1, Sma 1; R1, EcoR1. R5 and B1 were used for construction of PVL 1394 and S1 and R1 were used for insertion of the PTP cDNA fragment. The open bar represents PTPγ sequence. **B** Sf9 cells were metabolically labelled for 4 h with ^{35}S-Met after infection with the PTPγ recombinant virus for the indicated times. 1 mg protein extract (from 10-cm tissue culture dish) from each sample was immunoprecipitated with anti-PTPγ Ab and analyzed in SDS-PAGE.

We have constructed the PTPγ expression plasmid using the authentic polyhedron initiation codon, and fusing the cDNA to the polyhedron sequence several aa downstream of its initiation codon. Such an approach has been reported to yield higher level of expression than proteins without fusion to the polyhedron sequence [35]. Therefore, the low level expression of PTPγ is likely due to its intrinsic nature rather than inappropriate initiation sequence. It is possible that this protein is not compatible with the SF9 cells. Alternatively its phosphatase activity may somehow interfere with the expression. Cell death apparently was not the reason for the low expression since no obvious difference in cytopathic effect caused by infection of the recombinant and wild-type viruses was observed.

Expression of gag-PTPγ Fusion Protein in Normal and src Transformed NIH3T3 and RAT1 Cells

Fusion to the *gag* sequence is commonly seen in retrovirus-transduced oncogenes including *ros*, *fps*, *yes*, *myc*, *erbA*, *abl*, *fms*, *kit*, and *raf* [24]. The function of the *gag* sequence is thought to provide an initiation codon and help to stabilize and form active oncoproteins [15, 29]. We have shown that IR and IGFR genes could be truncated and fused to *gag* to form active oncogenes [23, 41]. Therefore, we thought there was a good chance to stably

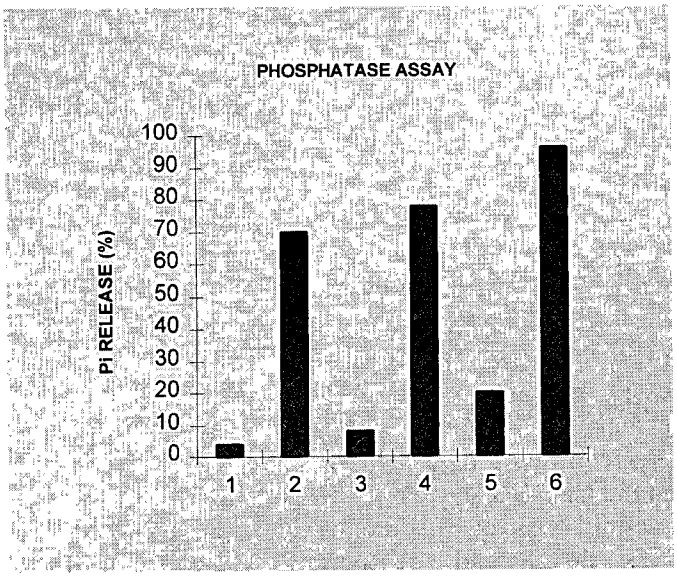


Fig. 4. Phosphatase assay: 1 mg protein of each cell lysate was immunoprecipitated with anti-PTPγ Ab. One-tenth of the immune complexes resuspended in PTPase reaction buffer was added to the ^{32}P-labelled Raytide peptide in the same buffer and incubated for 15 min at 37°C (see Materials and Methods). The free phosphate (Pi) was separated by paper chromatography and counted in scintillation counter. Pi release (%) = cpm (free Pi)/cpm (Raytide + free Pi). 1 = *src*/NIH3T3 cells; 2 = gag-PTPγ expressing *src*/NIH3T3 cells, 3 = *src*/Rat1 cells; 4 = gag-PTPγ expressing *src*//Rat1 cells; 5 = SF9 cells; 6 = SF9 cells infected with recombinant virus for 2 days.

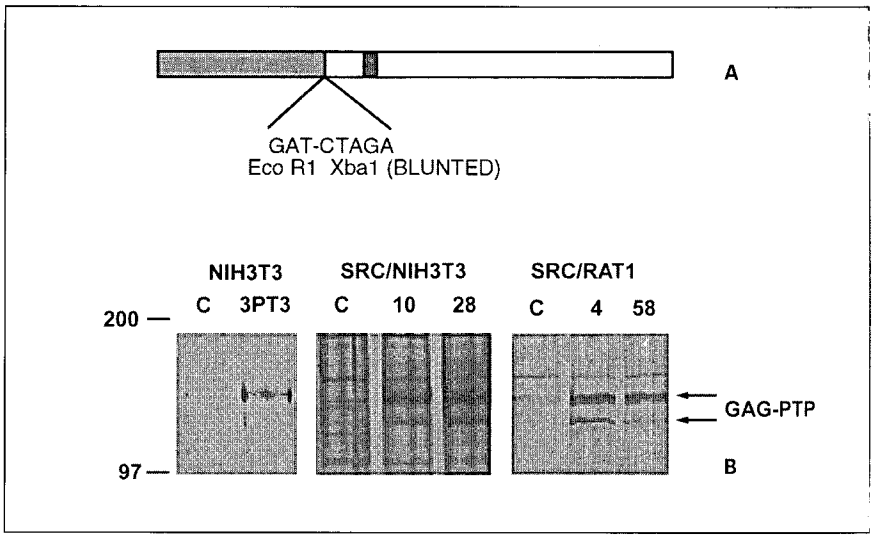


Fig. 5. gag-PTPγ expression in normal and *src*-transformed NIH 3T3 and Rat1 cells. **A** Structure of gag-PTPγ fusion cDNA. Hatched bar represents *gag* sequence of UR2; open bar represents PTPγ sequence, and filled bar represents its TM domain. The restriction sites used for cloning and the fusion junction are shown. **B** NIH3T3, *src*/NIH3T3 and *src*/Rat1 cells were transfected with gag-PTPγ expression vector and pSV2-Neo plasmid. After drug selection, resistant clones were isolated, amplified and analyzed for gag-PTPγ expression by metabolic labelling with ^{35}S-Met and immunoprecipitation with anti-PTPγ Ab. Representative clones and a control pSV2-Neo transfected clone are shown.

Table 1. Colony formation of GAG-PTP expressing cells

Clones	Colony No.	Clones	Colony No.
src/3T3-1	230	PTP/src/3T3-1	274
src/3T3-2	523	PTP/src/3T3-2	415
src/Rat-1	150	PTP/src/Rat-1	130
src/Rat-2	218	PTP/src/Rat-2	212

Parental *src* transformed NIH3T3 (src/3T3) and Rat1 (src/Rat) clones and the GAG-PTP expressing clones were seeded at 2,000 cells per 6-cm dish. Colonies were counted after 3 weeks of incubation.

express PTPγ as gag-PTP fusion protein in mammalian cells. The PTPγ cDNA fragment including the TM and cytoplasmic domains (aa 679–1,422) was linked to the *gag* sequence of UR2 to form a gag-PTP coding sequence in the mammalian expression vector pECE (fig. 5A). The plasmid was cotransfected with the pSV2-Neo into NIH3T3 cells and a number of stable expressing clones were isolated. The result showed that the PTPγ expression levels in those clones were very low (fig. 5B). Since PTPγ was suggested to be a candidate of tumor suppressor based on its chromosomal localization [19] and because of its presumed activity against PTKs, *src*-transformed NIH3T3 and Rat1 cell lines were used to establish stable gag-PTPγ expressing lines and to analyze the effect of PTPγ. Several clones from both *src*/NIH3T3 and *src*/Rat1 cells were established and shown to express a protein at the anticipated molecular weight as detected by metabolic labelling and immunoprecipitation with anti-PTPγ Ab (fig. 5B). Subcellular fractionation showed that most of the gag-PTPγ in 3T3 cells cofractionated with the P100 membrane component, and PTPγ could be detected on cell surface by biotin labeling of intact cells (data not shown). Phosphatase assays confirmed that this fusion protein was able to dephosphorylate Raytide, a peptide substrate (fig. 4, lanes 1–4). The low expression level of PTPγ in mammalian cells is consistent with that in insect cells and suggests that its low level of expression is likely due to its intrinsic property.

To detect whether gag-PTPγ can reverse the transformed phenotypes of *src*/NIH3T3 and *src*/Rat1 cells, colony formation assay was performed. The result showed that gag-PTP expressing cells gave rise to similar numbers of colonies as the parental cells, although the colony numbers varied among individual clones (table 1). To determine cell growth rate, parental *src*/NIH3T3 and *src*/Rat1 clones and their gag-PTPγ expressing derivative clones were seeded in 24-well plates. After incubation in 5% serum for 24 h, cells were grown in 0.5% serum and cell numbers were counted after 1 and 3 days of further incubation. No difference was observed among gag-PTP expressing cells and parental cells either (data not shown).

PTPs can regulate cell growth positively or negatively. PTP1B can suppress transformation by *neu* oncogene in PTP1B expressing NIH3T3 cells [5]. Microinjection of PTP1B into Xenopus oocytes demonstrated that it could block the maturation induced by insulin [38]. The SH2 domain containing PTP, PTP1C, inhibits cytokine-in-

duced mitogenic response in hematopoietic cells [17] whereas another SH2-containing PTP, Syp, is required for signal transduction mediated by a number of RPTKs and thus positively regulates cell growth [10]. Expression of a receptor-type PTP, CD45, in C127 murine cells has been shown to inhibit ligand-dependent phosphorylation of IGFR and PDGFR and their mitogenic response to IGF-1 and PDGF, respectively [25]. Conversely, expression of PTPα in rat fibroblast could dephosphorylate c-Src resulting in its activation and cell transformation [44]. Since transformation by *src* requires its continuous PTK activity, our result indicates that expression of PTPγ is unable to inactivate the $pp60^{src}$ or to block phosphorylation of its essential substrate(s) for cell transformation. This could be due to low expression level of the gag-PTPγ which is insufficient to overcome the activity of Src. Alternatively, the specificity of PTPγ or its compartmentalization may have precluded its interaction with Src or its substrates even though that gag-PTPγ appears to be associated with plasma membrane where functional Src is known to be localized and to exert its function.

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