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# Cell Surface Glycoprotein PZR Is a Major Mediator of Concanavalin A-induced Cell Signaling\*

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**PZR is an immunoglobulin superfamily cell surface protein containing a pair of immunoreceptor tyrosine-based inhibitory motifs. As a glycoprotein, PZR displays a strong association with concanavalin A (ConA), a member of the plant lectin family. Treatment of several cell lines with ConA caused tyrosine phosphorylation of a major cellular protein. Immunoblotting and immunoprecipitation studies indicated that this protein corresponded to PZR. Tyrosine phosphorylation of PZR was accompanied by recruitment of SHP-2 and was inhibited by PP1, a selective inhibitor of the Src family tyrosine kinases. Furthermore, c-Src was constitutively associated with PZR and was activated upon treatment of cells with ConA. Moreover, tyrosine phosphorylation of PZR was markedly enhanced in v-Src-transformed NIH-3T3 cells and was predominant in *Escherichia coli* cells co-expressing c-Src. Expression of an intracellular domain-truncated form of PZR in HT-1080 cells affected cell morphology and had a dominant negative effect on ConA-induced tyrosine phosphorylation of PZR, activation of c-Src, and agglutination of the cells. Together, the data indicate that PZR is a major receptor of ConA and has an important role in cell signaling via c-Src. Considering the various biological activities of ConA, the study of PZR may have major therapeutic implications.**

*in vitro* and inhibits tumor development *in vivo* (8, 9). Other activities of ConA include inhibition of the endocytosis of adrenergic receptors (10, 11) and induction of apoptosis (12). Despite these major biological activities, the potential uses of ConA and other plant lectins as therapeutic drugs are tempered by their lack of specificity. In addition, the precise mechanism by which these lectins function to regulate adhesion and proliferation is not clear.

Protein tyrosine phosphorylation plays a pivotal role in cell proliferation, differentiation, and transformation (13, 14). It is the initial step in cell signaling induced by extracellular stimuli, including growth factors, cytokines, antigens, and extracellular matrix. Tyrosine phosphorylation alters enzymatic activity and/or provides binding sites for downstream signaling molecules containing SH2 domains and PTB domains (15). Because these domains bind with specific tyrosine-phosphorylated motifs, tyrosine kinase signaling pathways gain specificity from the intrinsic binding preferences of these domains for short sequences that flank phosphotyrosine. Recently, we have isolated a cell surface glycoprotein designated PZR. PZR is a widely expressed member of the immunoglobulin superfamily (16). Its extracellular segment has significant sequence homology to myelin P0, a major structural protein of the myelin sheath. Its intracellular portion has two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that specifically interact with SHP-2 (17), an SH2 domain-containing tyrosine phosphatase with a crucial role in cell signaling (18). Tyrosine phosphorylation of PZR is observed upon treatment of cells with the tyrosine phosphatase inhibitor pervanadate and in cells over-expressing the catalytically inactive form of SHP-2 but not in cells treated with growth factors such as epidermal growth factor, platelet-derived growth factor, and insulin (16, 19). Although PZR is a potential receptor, its ligand is not known. Because PZR is a heavily glycosylated protein and it binds tightly with wheat germ agglutinin *in vitro* (16), we thought that a lectin family protein might serve as a ligand of PZR. In the present study, we demonstrate that PZR is a major mediator of cell signaling induced by ConA.

It has been generally accepted that complex carbohydrates play an important role in the regulation of cell adhesion and cell proliferation. This was demonstrated by the use of plant lectins that cross-link cell surface glycoproteins thereby initiating various cellular responses (1, 2). Concanavalin A (ConA)<sup>1</sup> is the most extensively investigated member of the lectin family of plant proteins (3, 4). It displays high affinities for terminal  $\alpha$ -D-mannosyl and  $\alpha$ -D-glucosyl residues (5). At physiological pH, ConA exists as a tetramer and displays cell-agglutinating and mitogenic activities (6, 7). More importantly, ConA has differential toxicity on normal and transformed cells

## EXPERIMENTAL PROCEDURES

**Materials**—HT-1080, HeLa, human embryonic kidney 293, NIH-3T3, and v-Src-transformed NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50  $\mu$ g/ml each of streptomycin and penicillin. Polyclonal and monoclonal anti-SHP-2 antibodies were obtained from Santa Cruz Biotechnology and Transduction Laboratory, respectively. The former was used for immunoprecipitation while the latter was employed for Western blotting. Monoclonal anti-phosphotyrosine 4G10 and anti-v-Src were purchased from Upstate Biotechnology Inc., and Oncogene Sciences, respectively. Tunicamycin, concanavalin A (ConA), succinyl-ConA, and ConA-agarose were from Sigma Chemical Co., whereas the Src family kinase inhibitor PP1 was from Calbiochem. Polyclonal anti-PZR serum 105 was raised in a rabbit against a GST fusion protein containing the

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<sup>1</sup> The abbreviations used are: ConA, concanavalin A; ITIM, immunoreceptor tyrosine-based inhibitory motif; GST, glutathione S-transferase; PBS, phosphate-buffered saline

intracellular domain of PZR (amino acids 191–269) as described previously (16). To enhance its specificity, the anti-serum was passed through a glutathione *S*-transferase-agarose column. Unless otherwise noted, the treated anti-serum was used.

**Generation of Stable HT-1080 Cell Lines with Altered PZR Expression**—Full-length PZR cDNA in sense and in antisense directions and an intracellular domain-truncated form (PZR<sub>X</sub>) corresponding to amino acids 1–199 of the PZR molecule were subcloned into the pCDNA3 vector (Invitrogen). The DNA constructs were used to transfect the human HT-1080 fibrosarcoma cell line by using the FuGENE6 cell transfection system (Roche Molecular Biochemicals). Clonal cell lines were isolated by G418 selection. Stable cell lines were maintained in complete culture medium supplemented with 0.25 mg/ml G418. Expression of both the full-length and the truncated form of PZR were analyzed by using anti-PZR serum 105.

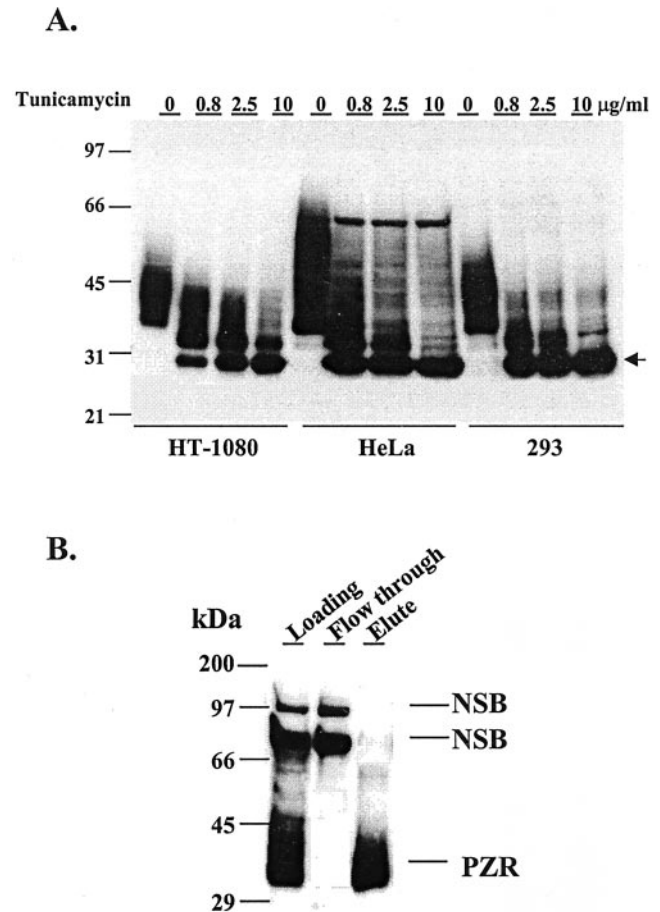
**Cell Stimulation, Extraction, Immunoprecipitation, and Western Blotting Analyses**—HT-1080, 293, and HeLa cells were cultured to 80–90% confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 units/ml each of penicillin and streptomycin. Cells were starved with 0% serum for 4 h before stimulation with ConA. The stimulation reactions were stopped by washing cells with ice-cold phosphate-buffered saline. The cells were lysed in buffer A containing 50 mM  $\beta$ -glycerophosphate (pH 7.3), 0.1 M NaCl, 5 mM EDTA, 1 mM EGTA, 5 mM  $\beta$ -mercaptoethanol, 1% Triton X-100, 0.2 mM  $\text{Na}_3\text{VO}_4$ , 0.1  $\mu\text{M}$  microcystin, 1.0 mM benzamide, 0.1 mM phenylmethylsulfonyl fluoride, 20  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{M}$  pepstatin A, and 1  $\mu\text{g}/\text{ml}$  aprotinin. Extracts were cleared by centrifugation. For immunoprecipitation, cell extracts were incubated overnight with various antibodies pre-bound to protein A-Sepharose. The beads were washed three times with an immunoprecipitation washing buffer containing 50 mM  $\beta$ -glycerophosphate (pH 7.3), 0.15 M NaCl, 2 mM EDTA, 1 mM EGTA, 5 mM  $\beta$ -mercaptoethanol, 0.1% Triton X-100, and 0.2 mM  $\text{Na}_3\text{VO}_4$ . For Western blot analyses, samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with various primary antibodies and were detected by using the ECL system with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Inc.).

**In Vitro Src Kinase Activity Assays**—HT-1080 cell extracts were subjected to immunoprecipitation with anti-v-Src antibody. The immunoprecipitates were washed with the aforementioned immunoprecipitation washing buffer and then with a Src kinase assay buffer containing 25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4 mM  $\text{MgCl}_2$ , 10 mM  $\text{MnCl}_2$ , 0.2 mM  $\text{Na}_3\text{VO}_4$ , and 2 mM dithiothreitol. The kinase assay system contained 50  $\mu\text{l}$  of the assay buffer plus 50  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (1000 cpm/pmol) and 0.2 mg/ml rabbit skeletal muscle enolase or glutathione *S*-transferase fusion protein carrying the intracellular portion of PZR. The reactions were run at room temperature for 20 min and stopped by adding SDS sample buffer. Phosphorylated products were detected by autoradiography after separation on 10% SDS gels.

**Tyrosine Phosphorylation of PZR in *E. coli* Cells Co-expressing *c-Src* and *SHP-2* Binding Assays**—The intracellular domain of PZR (amino acid residues 192–269) and the correspondent Tyr-to-Phe mutant form PZR(Y241F/Y263F) were constructed as GST fusion proteins by using the pGex-2T vector, which contains an ampicillin-resistant gene. The fusion proteins were expressed in DE3(LysS) *Escherichia coli* cells co-expressing *c-Src* carried by a pET9a vector, which is kanamycin-resistant. Upon treatment with 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside at 37 °C for 3.5 h, the cells were harvested and extracted in PBS by sonication. The extracts were subjected to separation on SDS gels and further to Western blot analyses. For purification of the fusion proteins, cell extracts were incubated with glutathione-Sepharose beads. After extensive washing with PBS, the beads were incubated with HT-1080 extracts for 2 h and were then washed as described for immunoprecipitation described above. Proteins bound to the beads were analyzed by Western blotting with specific antibodies.

## RESULTS

**PZR Is a Glycoprotein and It Binds ConA**—The PZR cDNA encodes a type I transmembrane protein of 269 amino acid residues. However, the PZR protein is highly heterogeneous on SDS gel with molecular sizes larger than the calculated value. For example, PZR from HeLa cells ran at 30–70 kDa whereas that from HT-1080 cells and 293 cells displayed proteins bands of 31–50 kDa (Fig. 1A). It is not known if this heterogeneity in the size of PZR is related to any specific type of cell transformation. Nonetheless, it is caused by different degrees of glyco-

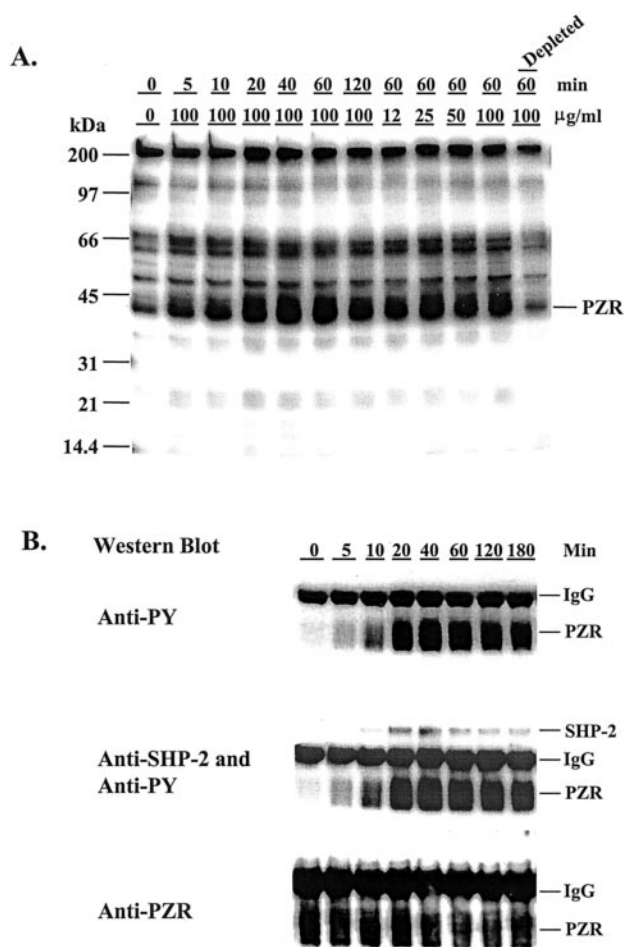


**FIG. 1. PZR is a heterogeneous glycoprotein, and it binds ConA.** A, HT-1080, HeLa, and 293 cells were treated with the indicated concentrations of tunicamycin for 48 h, and cells extracted were subjected to Western blotting analysis with anti-PZR antibody 105. The position of non-glycosylated PZR is indicated by an arrow. Note the different sizes of PZR in non-treated cells. B, HT-1080 cell extracts were loaded onto a ConA-agarose column. After being washed with the cell extraction buffer, the column was eluted with 0.3 M *N*-acetylglucosamine. The loading material, flow-through, and elute were subjected to Western blot analysis with non-treated anti-PZR antibody. NSB denotes nonspecific bands detected by the non-treated antibody.

sylation, because treatment of cells with tunicamycin resulted in a sharp PZR band of ~30 kDa, which agrees with the molecular size predicted from its primary structure (Fig. 1A). Tunicamycin inhibited *N*-linked glycosylation, and thus the data suggest that *N*-linked glycosylation is responsible for the heterogeneous size of PZR. In fact, PZR contains two putative *N*-linked glycosylation sites in its extracellular region and *in vitro* deglycosylation by *N*-glycosidase F also resulted in a sharp band of ~30 kDa (16). As a glycosylated protein, PZR is a potential receptor of the lectin family proteins. Our earlier studies have showed that PZR binds wheat germ agglutinin (16). To further study the interaction of PZR with the lectin family proteins and its biological functions, we employed ConA, the most extensively studied lectin family of plant proteins. As shown in Fig. 1B, when HT-1080 cell extracts were passed through a ConA-agarose column in the presence of 0.3 M NaCl, PZR was totally depleted from cell extracts. Consequently, all the bound PZR was recovered by eluting the column with 0.3 M *N*-acetylglucosamine.

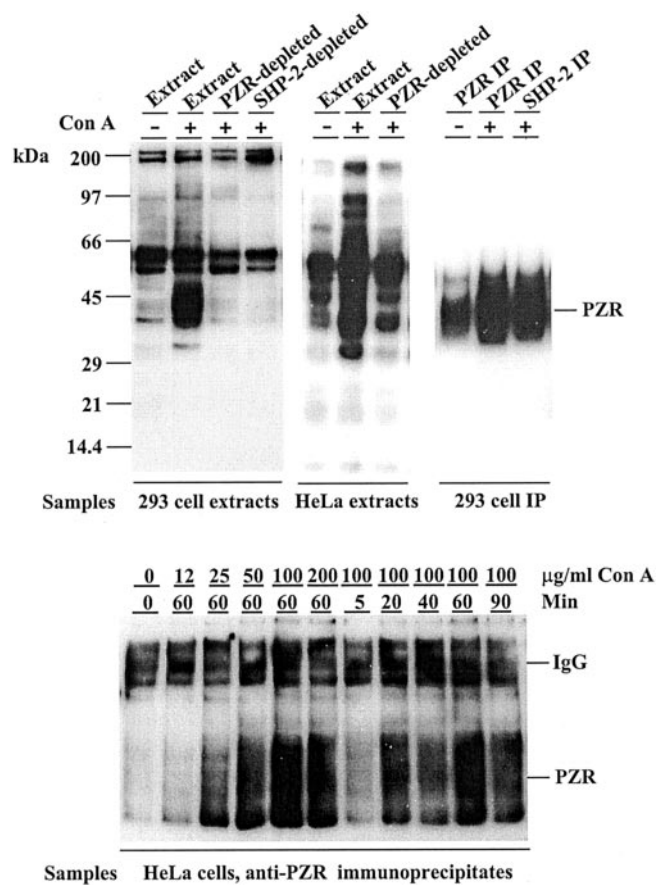
**ConA Induces Tyrosine Phosphorylation of PZR and Recruitment of *SHP-2***—ConA exists in tetrameric and higher oligomeric forms under physiological pH. Because of its ability to cross-link cell surface glycoproteins, ConA exhibits multiple biological activities, but the signal transduction mechanisms





**FIG. 2. PZR is a major protein that is phosphorylated on tyrosine residues upon treatment of HT-1080 cells with ConA.** HT-1080 cells were serum-starved for 2 h and then treated with variable doses of (0–100  $\mu\text{g}/\text{ml}$ , A) or a fixed dose (100  $\mu\text{g}/\text{ml}$ , B) of ConA for 0–180 min as indicated. Cell extracts (A) or anti-PZR immunoprecipitates (B) were analyzed by Western blotting with anti-phosphotyrosine (A), anti-SHP-2, and anti-PZR as indicated. In the last lane of A on the right, PZR in cell extracts was depleted by anti-PZR antibody.

are not well understood. Initially, we analyzed the tyrosine phosphorylation of cellular proteins upon treatment of HT-1080 cells with ConA. When serum-starved HT-1080 cells were treated with 100  $\mu\text{g}/\text{ml}$  ConA, clear tyrosine phosphorylation of a major protein around 43 kDa was observed after 20 min (Fig. 2A). The same phosphorylated band was seen in cells treated with 12  $\mu\text{g}/\text{ml}$  ConA for 1 h. The protein co-migrated with PZR, and immunoprecipitation of the cell extracts with anti-PZR antibody caused total depletion of the tyrosine-phosphorylated protein from the cell extracts, indicating the protein corresponds to PZR (the far right lane in Fig. 2A). Anti-phosphotyrosine Western blot analysis of the PZR immunoprecipitates further verified these results (Fig. 2B). Tyrosine phosphorylation of PZR appeared after 5 min of ConA treatment and reached a plateau after 40 min. It should be pointed out that immunodepletion and immunoprecipitation experiments with preimmune PZR serum and a irrelevant rabbit polyclonal anti-LAIR-1 antibody demonstrated that the detection of PZR was highly specific (data not shown but provided for scrutiny by reviewers). Our earlier studies have shown that PZR is a major anchor protein of SHP-2 on the cell membrane (16). As expected, ConA-induced tyrosine phosphorylation of PZR was accompanied by recruitment of SHP-2. The level of SHP-2 binding is directly proportional the level of tyrosine phospho-

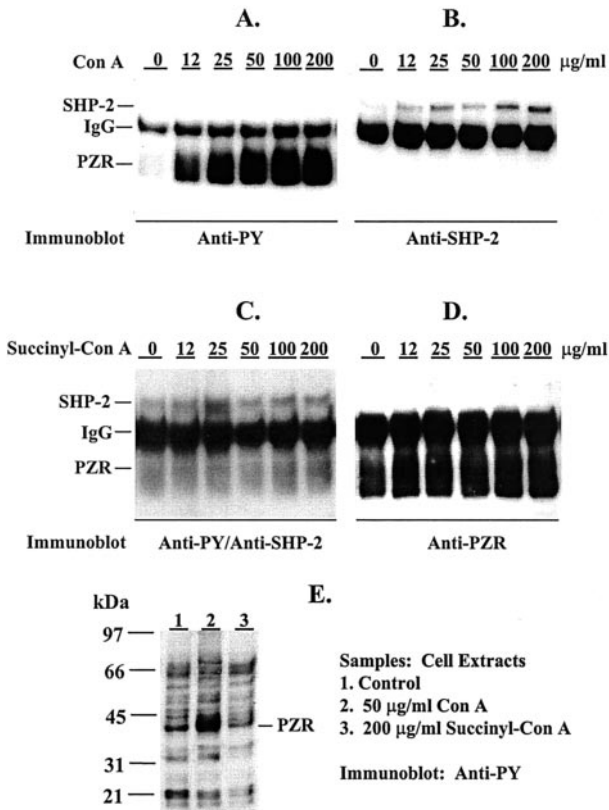


**FIG. 3. PZR is a major protein that is phosphorylated on tyrosine residues upon treatment of 293 and HeLa cells with ConA.** HeLa and 293 were serum-starved for 2 h and then treated with a fixed dose (100  $\mu\text{g}/\text{ml}$ , indicated by “+” in the upper panel) or variable doses (0–200  $\mu\text{g}/\text{ml}$ , lower panel) of ConA for 60 min (upper panel) or 0–90 min as indicated (lower panel). Cell extracts and immunoprecipitates obtained with anti-PZR or anti-SHP-2 as indicated were analyzed by Western blotting with anti-phosphotyrosine. Note that the SDS gel electrophoresis in the lower panel was performed under non-reducing conditions so that IgG runs an intact molecule of over 150 kDa.

rylation of PZR. This is reminiscent of growth factor-induced tyrosine phosphorylation of receptors and recruitment of SH2 domain-containing signaling proteins. However, it should be noted that no significant tyrosine phosphorylation of SHP-2 was observed. To demonstrate that induction of PZR tyrosine phosphorylation is a general phenomenon in ConA-treated cells, we studied several other cell lines. Fig. 3 shows the results obtained with 293 and HeLa cells. Tyrosine phosphorylation of PZR was verified by immunodepletion and immunoprecipitation. The time course and the dose response of the phosphorylation in these cells were similar to those observed in HT-1080 cells. Immunoprecipitation of SHP-2 also caused total depletion of tyrosine-phosphorylated PZR from 293 cell extracts, indicating that all the tyrosine-phosphorylated PZR was associated with SHP-2 in ConA-treated cells. Note that PZR from HeLa cells showed as heterogeneous bands of 30–70 kDa on SDS gel. This is due to a different degree of glycosylation.<sup>2</sup> We also observed similar tyrosine phosphorylation of PZR in HepG2 and A431 cells (not shown), suggesting that ConA-induced tyrosine phosphorylation of PZR is a general phenomenon.

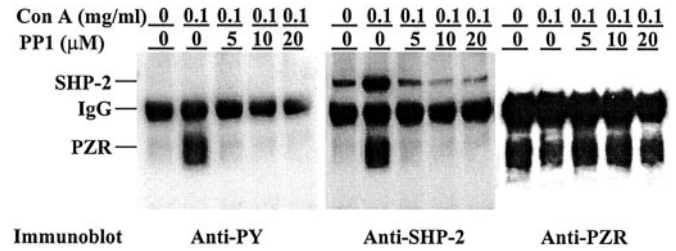
*Bivalent Succinyl-ConA Failed to Induce Phosphorylation of PZR*—ConA exists as a tetramer, between pH 5.8 and pH 7.0.

<sup>2</sup> R. Zhao, A. Guerrah, H. Tang, and Z. J. Zhao, unpublished data.



**FIG. 4. Succinyl-ConA fails to induce tyrosine phosphorylation of PZR.** Serum-starved HT-1080 cells were treated with 0–200 µg/ml ConA or succinyl-ConA for 1 h as described in Fig. 2. Cell extracts were immunoprecipitated with anti-PZR. The immunoprecipitates (A–D) and cell extracts (E) were analyzed by Western blotting with anti-phosphotyrosine, anti-SHP-2, and anti-PZR as indicated. Equivalent amounts of samples were used in A, B, C, and D. Equal amounts of total proteins were loaded in E.

It forms higher aggregates above pH 7.0. Much of the biological activity (e.g. agglutinating and mitogenic activity) of ConA depends on its degree of aggregation. Succinylation results in a dimeric form that remains as a dimer above pH 5.6 (3). Succinyl ConA still binds ConA receptors but is ineffective at cross-linking them and therefore fails to exhibit any biological activity of ConA. We thus compared the effects of ConA and succinyl ConA on tyrosine phosphorylation of PZR and its association with SHP-2 (Fig. 4). Unlike ConA, which caused clear tyrosine phosphorylation of PZR and recruitment of SHP-2 at a concentration of 12 µg/ml (Fig. 4, A and B), succinyl ConA failed to induce either of these at a concentration as high as 200 µg/ml (Fig. 4, C and E). For simplicity, Fig. 4C shows results of immunoblotting with anti-PY and anti-SHP-2 together. Separated immunoblotting of anti-PZR immunoprecipitates with anti-phosphotyrosine and anti-SHP-2 gave rise to the same negative results. Fig. 4C represents a relatively overexposed Western blot, and thus the basal tyrosine phosphorylation of PZR and its association with SHP-2 became evident. Fig. 4D shows equal immunoprecipitation of PZR by anti-PZR antibody. Because ConA is tetraivalent, i.e. it has four sugar binding sites, the data seem to suggest that tetramerization of PZR or clustering with other proteins is required for the induction of tyrosine phosphorylation. However, it should be noted that the proper position of each receptor subunit in a dimer is also crucial for induction of signaling. It is possible that bivalent succinyl-ConA also binds cell surface PZR, but spatial restraints render it unable to cross-link two PZR molecules, or that PZR dimers formed by bivalent ConA are not in the right



**FIG. 5. Src-family tyrosine kinase inhibitor PP1 blocks ConA-induced tyrosine phosphorylation of PZR and its association with SHP-2.** HT-1080 cells were pre-treated with the indicated concentrations of PP1 for 1 h before stimulation of the cells with 100 µg/ml ConA for 1 h. Western blot analysis of anti-PZR immunoprecipitates was performed as described in Fig. 2.

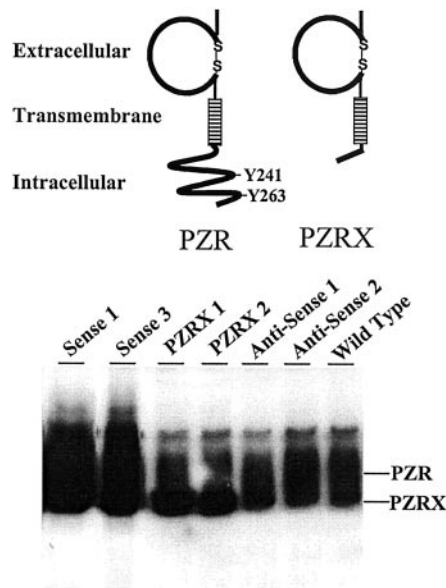
conformation to cause tyrosine phosphorylation. In any case, ConA is a useful tool for studying PZR.

*Src Family Tyrosine Kinases Are Involved in Tyrosine Phosphorylation of PZR Induced by ConA*—Src family protein-tyrosine kinases play a crucial role in cell adhesion. To investigate possible involvement of these kinases in phosphorylation of PZR, we pre-treated HT-1080 cells with the Src family tyrosine kinase inhibitor PP1. PP1 at a concentration of 5 µM totally inhibited tyrosine phosphorylation of PZR and its association with SHP-2 (Fig. 5). Note the tyrosine phosphorylation of PZR and the association with SHP-2 at the basal level. Although PP1 is not a very specific Src family kinase inhibitor, the results at least suggest that a Src family tyrosine kinase may be responsible for phosphorylation of PZR. The association of PZR with c-Src further supports this notion. As shown in Fig. 6A, c-Src was detected in the immunoprecipitates of PZR in non-treated as well as ConA-treated cells. Compared with the level of c-Src present in anti-c-Src immunoprecipitates, c-Src co-immunoprecipitated with PZR represented a small but evident portion of total c-Src in cell extracts. Note that control experiments with preimmune anti-PZR serum demonstrated the specificity of the immunoprecipitation. Because anti-PZR and anti-c-Src antibodies used in this study were able to deplete PZR and c-Src, respectively, from cell extracts, we estimated that about 2–4% of c-Src was constitutively associated with PZR. We further analyzed the activity of c-Src by using the commonly used rabbit muscle enolase as a substrate. The data shown in Fig. 6B demonstrated that treatment of cells with ConA resulted in increased c-Src activity. Quantification of the radioactive bands by scintillation counter revealed about 3-fold increase of Src kinase activity over the basal level. Similar activation of c-Src was observed when a GST fusion containing the intracellular part of PZR was used as a substrate. Taken together, the data indicate that c-Src may be at least partly responsible for tyrosine phosphorylation of PZR induced by ConA.

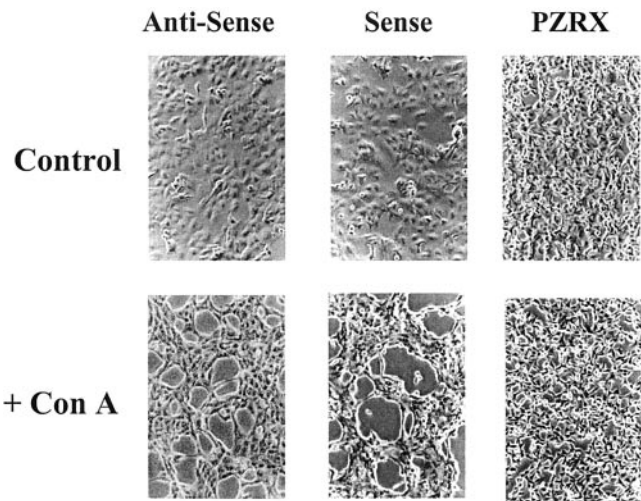
*PZR Is Hyper-phosphorylated in v-Src-transformed NIH-3T3 Cells and in E. coli Cells Co-expressing c-Src*—To further verify the involvement of Src family kinases in phosphorylation of PZR, we analyzed the phosphorylation of PZR in v-Src-transformed NIH-3T3 cells with normal NIH-3T3 cells as control. As shown in Fig. 7A, a very strong tyrosine phosphorylation of PZR was observed in the v-Src-transformed cells. Note that a much lower basal level of PZR phosphorylation was seen in the non-transformed control cells and that phosphorylation of PZR was accompanied by binding of SHP-2. Immunoblotting analyses with anti-PZR antibody revealed that control and v-Src-transformed cells expressed comparable amounts of PZR. Oncogenic v-Src is a deregulated, constitutively active form of c-Src. These data thus provide further evidence that Src family tyrosine kinase is at least partly responsible for in tyrosine







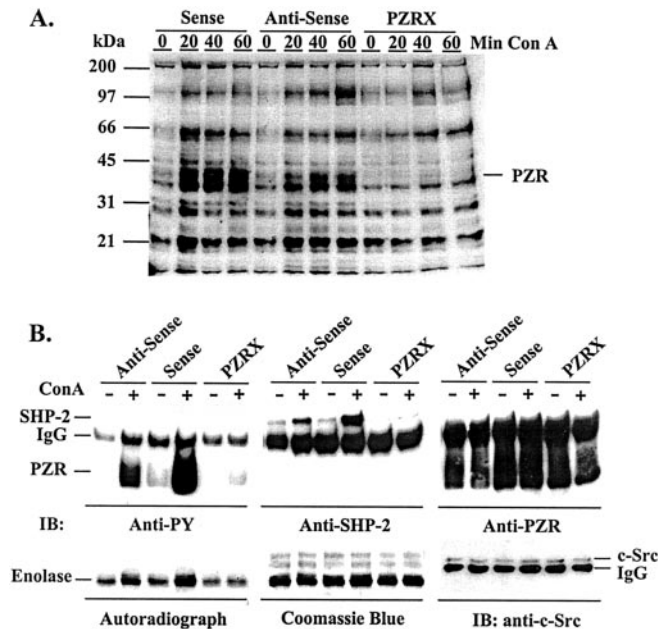
**FIG. 8. Overexpression of full-length and intracellular domain-truncated forms of PZR in HT-1080 cells.** The upper panel shows a schematic diagram of the PZR constructs. The bottom panel shows the expression level of PZR and PZR<sub>X</sub> in stable HT-1080 cell lines transfected with PZR<sub>X</sub> and PZR in sense and in antisense orientations. The levels of PZR in the antisense cells and in the wild type cells were essentially the same. PZR<sub>X</sub> runs slightly ahead of PZR on SDS gel. Note that cells expressing the truncated PZR also express full-length PZR.



**FIG. 10. ConA-induced agglutination of HT-1080 cells is enhanced by overexpression of full-length PZR but blocked by expression of the intracellular domain-truncated PZR<sub>X</sub>.** Near confluent HT-1080 cells expressing antisense PZR control, sense PZR, or PZR<sub>X</sub> were serum-starved for 2 h and then were treated with 100  $\mu$ g/ml ConA at 37  $^{\circ}$ C for 2 h. Phase contrast photos were taken with  $\times 100$  magnification. Representative data of four independent experiments with eight different stable cell lines are shown.

also expressed the endogenous full-length form of PZR, which can be immunoprecipitated by anti-PZR antibody (Fig. 9B, right panel). The increased tyrosine phosphorylation of PZR in the sense PZR-expressing cells is attributable to a higher PZR expression, but the loss of PZR phosphorylation in PZR<sub>X</sub>-expressing cells was not caused by a decrease in PZR protein level in comparison with the antisense control cells. The blocking of tyrosine phosphorylation by expression of the truncated form indicates that the latter form has a dominant negative role. This also provides direct evidence that activation of PZR requires clustering of PZR molecules with the functional intracellular part. This is reminiscent of the dominant negative effects observed with the kinase mutant of growth factor receptors (20). We also analyzed the activation of c-Src in these stable cell lines (Fig. 9B, bottom panels). As expected, expression of the truncated form of PZR also abolished ConA-induced activation of c-Src. Together, the data imply that ConA causes cross-linking of cell surface PZR and thereby results in activation of c-Src which in turn phosphorylates PZR. Of course, we cannot rule out involvement of other cell surface glycoproteins and other tyrosine kinases.

**PZR Mediates ConA-induced Agglutination of HT-1080 Cells**—By clustering cell surface glycoproteins, one of the major activities of ConA is to cause agglutination of cells. To examine whether PZR plays a role in this process, we treated the stable HT-1080 cell lines with ConA and investigated cell morphology changes (Fig. 10). Cells overexpressing the full-length form of PZR showed much enhanced agglutination in comparison with the antisense control cells. In contrast, expression of the truncated form totally blocked the process. Because agglutination of the adherent cells involves cell detachment and migration, the data suggest an important role of PZR in these processes. Tyrosine phosphorylation of PZR is presumably essential for the detachment and migration. However, treatment of cells with Src kinase inhibitor PP1 totally blocked tyrosine phosphorylation of PZR but only slightly slowed down the agglutination process (data not shown). This is probably due to the fact that PP1 itself causes detachment of the cells from the culture plates and suspended cells are more easily to be agglutinated by ConA. Finally, it should be noted that overexpression of PZR<sub>X</sub> *per se* had significant effects on the morphologies of



**FIG. 9. Overexpression of an intracellular domain-truncated PZR blocks ConA-induced tyrosine phosphorylation of PZR and activation of c-Src.** HT-1080 cells transfected with sense PZR, antisense PZR, or intracellular domain-truncated PZR<sub>X</sub> were serum-starved for 2 h and then left untreated or treated with 100  $\mu$ g/ml ConA for 20–60 min (upper panel) or for 60 min (lower panel). Cell extracts (upper panel) and anti-PZR and anti-c-Src immunoprecipitates (lower panel) were subjected to Western blotting analysis with anti-phosphotyrosine, anti-SHP-2, and anti-c-Src or to Src kinase activity assays followed by autoradiography. Representative data of four independent experiments with eight different stable cell lines are shown.

expressing the truncated form of PZR. As a consequence of loss of tyrosine phosphorylation, binding of SHP-2 was also abolished in cells expressing the truncated form of PZR. It should be pointed out that cells expressing the truncated form of PZR

HT-108 cells (Fig. 10, upper panel). The PZR-expressing cells appeared more transformed as indicated by their spindle-like structures. In contrast, PZR-overexpressing cells and antisense control cells displayed flattened cells morphology. The spindle shape of the PZR cells suggests possible loss of cell contact inhibition. Further study is underway to investigate the effects of PZR on cell tumorigenicity.

#### DISCUSSION

In the present study, we demonstrated that PZR is a major protein that is phosphorylated on tyrosine residues upon treatment of cells with ConA. Furthermore, ConA causes phosphorylation of PZR by cross-linking cell surface PZR molecules, which is similar to the manner by which growth factors or cytokines activate their receptors. The most compelling evidence for this comes from the fact that an intracellular domain-truncated form of PZR has a dominant role in blocking ConA-induced PZR activation. Therefore, it is fair to say that PZR is a major receptor of ConA. Although ConA may also bind other cell surface glycoproteins and induce their activation, PZR is by far the major tyrosine-phosphorylated protein in at least three cell lines examined in this study. This is probably due to the fact that PZR is a relatively abundant cell surface molecule. In fact, our earlier studies indicate that depletion of PZR causes depletion of nearly 50% of SHP-2 in cell extracts (16). As a major receptor of ConA, PZR must be an important transducer of ConA-induced cell signaling. Indeed, our studies indicated that cross-linking of PZR by ConA lead to activation of c-Src, which may be responsible for phosphorylation of PZR and possibly other proteins. Phosphorylation of PZR in turn recruits SHP-2, which by itself is an essential signal transducer. Further downstream signaling events still need to be investigated.

ConA has multiple biological activities dependent on cell types. Importantly, it displays differential toxicity toward normal and tumor cells (8, 9). However, the potential uses of ConA as therapeutic drugs are tempered by its nonspecificities and by the lack of understanding of its functional mechanisms. Here we demonstrated that PZR is a major receptor of ConA thereby providing a specific target for further investigation. In this study, we focused on ConA, the most extensive studied lectin protein. We believe other lectin proteins may also use PZR as a receptor. Our earlier studies have shown that PZR also binds wheat germ agglutinin (16). Different lectins have different binding affinities and selectivity to carbohydrates, and thus the biological effects may differ. In addition, although lectin family proteins were originally identified in plants, mammalian cells also contain a large numbers of proteins with lectin domains (21–24). Whether any of these proteins specifically interact with PZR is not known. PZR is a highly heterogeneous protein due to different degrees of glycosylation, and PZR proteins from different human cells displayed distinct mobilities on SDS gels. For example, PZR in HeLa cells runs as a much broader band than that found in 293 and HT-1080 cells (see Fig. 3). This indicates that transformed cell lines may have different modification of PZR. It is not known whether normal cells differ from transformed cells in the glycosylation of PZR. If so, PZR might be an excellent target for therapeutic drug development to treat cancer. Finally, because ConA induces activation of PZR by cross-linking the protein on the cell surface, the action of ConA can be mimicked specifically by anti-PZR antibodies that recognize the extracellular segment of PZR. In an earlier study, we have shown that a specific antibody can induce tyrosine phosphorylation of LAIR-1, an ITIM-containing receptor expressed in hematopoietic cells (25). Therefore, PZR antibodies may have potential for therapeutic applications.

PZR belongs to ITIM-bearing cell surface receptors. ITIMs

were defined as inhibitory motifs, because they were initially found in inhibitory immunoreceptors like Fc $\gamma$ RIIB and KIR and they mediate the inhibitory effects of these proteins on signal transduction (26, 27). Furthermore, because phosphorylation of the tyrosyl residue in the ITIMs triggers binding and activation of tyrosine phosphatases SHP-1 and SHP-2 and the inositol phosphatase SHIP, the inhibitory functions of ITIMs are thought to be executed by these phosphatases. SHP-2 is a widely distributed intracellular protein-tyrosine phosphatase containing SH2 domains. Studies have shown that SHP-2 plays a crucial role in cell signaling, because expression of its catalytically inactive Cys-to-Ser mutant form blocks growth factor-induced activation of mitogen-activate protein kinase (18, 28). Furthermore, disruption of the mouse *shp-2* gene caused death of mouse embryos at mid-gestation (29). Further studies with cells derived from SHP-2-deficient mice demonstrated impairment in erythropoiesis and cell migration (30, 31). However, the mechanism by which SHP-2 functions is still poorly understood, and we think that PZR may be involved in this function. Purified SHP-2 possesses low activity *in vitro* due to internal structural suppression (32, 33). Therefore, SHP-2 can be viewed as an inactive enzyme in the resting state of cells, which is activated by binding through SH2 domains to tyrosine-phosphorylated receptors on cell membrane. As a major anchor protein of SHP-2, PZR may primarily serve as an activator SHP-2, although it can also serve as a substrate (17). As a phosphatase, the main function of SHP-2 is certainly dephosphorylation, and this can have positive or negative effects on signaling processes. The net effect of SHP-2 in a cell signaling process is dependent on the targets it dephosphorylates. By studying the PZR-controlled activation of SHP-2 and the cell signaling events that are initiated, we should gain further insight into the function of SHP-2.

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