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EVIDENCE AGAINST DEPHOSPHORYLATION OF INSULIN-ELICITED PHOSPHOTYROSINE PROTEINS IN VIVO BY THE PHOSPHATASE PTP2C

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Summary: In order to determine whether the tyrosine phosphatase PTP2C dephosphorylates insulin-elicited phosphotyrosine proteins in vivo, we have compared the patterns of protein tyrosine phosphorylation and its reversal in the kidney 293 cell line with those in 293 cell lines overexpressing PTP2C and a catalytically inactive point mutant of PTP2C. In all three cell types insulin caused the rapid tyrosine phosphorylation of a 160 kD protein, which was shown not to be the insulin receptor substrate 1 (IRS-1) and may be the recently described IRS-2, as well as that of a 100 kD polypeptide, which is probably a mixture of the β subunits of the insulin and insulin-like growth factor I receptors. There was no difference among the three cell lines in the extent of tyrosine phosphorylation or in the rate of its reversal upon insulin withdrawal. These results indicate that PTP2C does not function to dephosphorylate these proteins significantly in vivo.

The insulin receptor is a tyrosine kinase that phosphorylates itself and target substrate proteins in response to insulin binding (1). A prominent substrate of the insulin receptor is the protein known as insulin receptor substrate 1 (IRS-1) . IRS-1 is phosphorylated on multiple tyrosine residues, which then serve as docking/effector sites for signaling proteins containing src homology 2 (SH2) domains (1,2). The receptor for insulin-like growth factor I (IGF I), which is homologous to the insulin receptor, similarly acts through phosphorylation of IRS-1 on the same tyrosine residues (3). A second substrate for phosphorylation by these receptors is likely to be the recently described protein designated IRS-2 (4,5). IRS-2 is an insulin-elicited phosphotyrosine

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Abbreviations used: IGF I, insulin-like growth factor I; IRS-1, insulin receptor substrate 1; Ptyr, phosphotyrosine; SH2, src homology 2.

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(Ptyr) protein, first detected in the liver and muscle of mice lacking IRS-1 and then characterized in the liver of normal mice (4,5). Although the sequence of IRS-2 and specific antibodies for IRS-2 have not yet been reported, IRS-2 has been shown to bind to two SH2 domain-containing proteins, phosphatidylinositol 3-kinase and Grb2, and so IRS-2 probably functions much like IRS-1.

Termination of insulin and IGF I signaling requires the tyrosine dephosphorylation of IRS-1 and IRS-2, as well as of the receptors. A phosphatase that is a candidate for this role is PTP2C (also referred to as Syp, SH-PTP-2, and PTP1D), which contains two SH2 domains in it amino terminal region. PTP2C has been found to bind via its SH2 domains to the Ptyr forms of IRS-1 and the insulin receptor and to dephosphorylate both these proteins (6-8). In order to assess whether PTP2C participates in terminating insulin and IGF-1 signaling in vivo, we have examined the effects of overexpression of PTP2C and of a catalytically inactive point mutant of PTP2C on insulin-elicited protein tyrosine phosphorylation in the kidney 293 cell line.

Materials and Methods

Cell lines. The 293 cell line that overexpresses PTP2C, one that overexpresses a catalytically inactive point mutant of PTP2C (459 Cys to Ser), and the control one that contains only the pRC/CMV vector (Invitrogen Corporation) were those described by Zhao et al. (9) Cells were grown on 10 cm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 0.5 mg/ml G418, 100 μg/ml streptomycin, and 100 units/ml penicillin. The medium was changed every other day. Cells were used upon reaching confluence. At this point there was 10-12 mg total cell protein per plate. Before use, the cells were washed with serum-free DMEM and incubated in serum-free DMEM for 2 h.

Cell treatment and lysis. In order to determine the time course for insulin-stimulated protein tyrosine phosphorylation, 10 cm plates were treated at 37° with 1 μM insulin in DMEM containing 100 μg/ml bovine serum albumin for various times. Each plate of cells was then lysed in 2 ml of ice-cold buffer consisting of 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1.5% Triton X-100, pH 7.0, containing phosphatase and protease inhibitors (100 mM NaF, 10 mM Na pyrophosphate, 1 mM EGTA, 1 mM Na orthovanadate, 10 μg/ml aprotinin, 10 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride). The lysates were cleared of insoluble material by centrifugation at 37,000 x g (max) for 1 h; and the supernatant was passed through a 0.22 micron filter (SLGVO25LS, Millipore) to remove any traces of insoluble material. Samples for sodium dodecylsulfate (SDS) gel electrophoresis were prepared from these supernatants by the addition of a 4X sample buffer containing 16% SDS and 40 mM dithiothreitol. The reversal of insulin-elicited tyrosine phosphorylation was examined by treating cells with 1 μM insulin for 5 min, as described above, then washing the plates rapidly two times with 10 ml DMEM, and incubating the cells in 10 ml DMEM at 37° for various times, after which cleared lysates were prepared as described above. In the experiment where IRS-1 was immunoprecipitated, the lysates (250 μl) were mixed with 12 μg affinity-purified antibodies against the carboxy terminal peptide of IRS-1 (10) or irrelevant rabbit IgG for 1 h, followed by 10 μl of protein A-Sepharose for 2 h. For determination of the amount of PTP2C in the 293 cell lines, 10 cm plates were lysed directly in 1 ml of SDS sample buffer with protease inhibitors.

Immunoblotting. SDS samples were run on 0.75 mm thick slab gels, and the proteins were transferred electrophoretically to ImmobilonP (Millipore) in 25 mM Tris, 192 mM glycine, 0.01% SDS, 20% methanol at 400 mA for 3 h at 4°. In the case of the immunoblots for Ptyr, the ImmobilonP membranes were treated with the RC20 monoclonal antibody conjugated to horseradish peroxidase according to the manufacturer's instructions (Transduction Laboratories), and the labeled polypeptides detected by the enhanced chemiluminescence method (ECL reagent, Amersham Life Sciences). In the case of the immunoblots for PTP2C and IRS-1, the blots were blocked with 5% Carnation non-fat dry milk in TBST (20 mM Tris, 150 mM NaCl, 0.3% Tween-20, pH 7,.6), treated with a 1/4000 dilution of rabbit antiserum against a truncated form of human PTP2C lacking the SH2 domains (11) or with 5 μg/ml antibodies against the carboxy terminal peptide of IRS-1 in 1% milk/TBST, washed with TBST, treated with goat antibodies against rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad Laboratories), and developed with the ECL reagent. For the PTP2C, pure recombinant human PTP2C (11) was used as a standard.

Results

In order to investigate the role of PTP2C in insulin-elicited protein tyrosine phosphorylation, we made use of three lines of stably transfected human embryonic kidney 293 cells that were generated by Zhao et al. (9) One overexpressed human PTP2C (designated PTP2Cwt); the second overexpressed the catalytically point mutant of PTP2C in which Cys 459 at the active site is replaced by Ser (designated PTP2Cmut); and the third contains only the vector used for transfection (designated vector). The actual amounts of PTP2C (normal or normal plus mutant) in these lines were determined by immunoblotting with recombinant PTP2C as the standard (Fig. 1). The amounts were approximately 120, 1000, and 600 ng PTP2C per mg total protein for the vector, PTP2Cwt, and PTP2Cmut, respectively. These values agree with the finding of

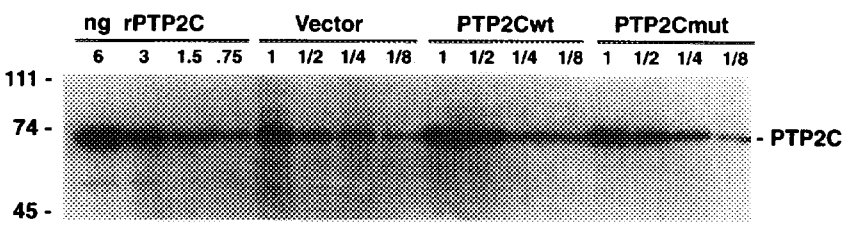

Figure 1. Amounts of PTP2C in 293 cell lines. Recombinant PTP2C (specified ng in the first four lanes) and lysates of the 293 cell lines described in the text (vector, PTP2Cwt, and PTP2Cmut) were immunoblotted for PTP2C. The lanes under Vector labeled 1, 1/2, 1/4, and 1/8 contained 0.25, 0.12, 0.06, and 0.03%, respectively, of a 10 cm plate. The corresponding lanes under PTP2Cwt and PTP2Cmut contained 1/5 these amounts (0.05% of a 10 cm plate and fractions thereof).

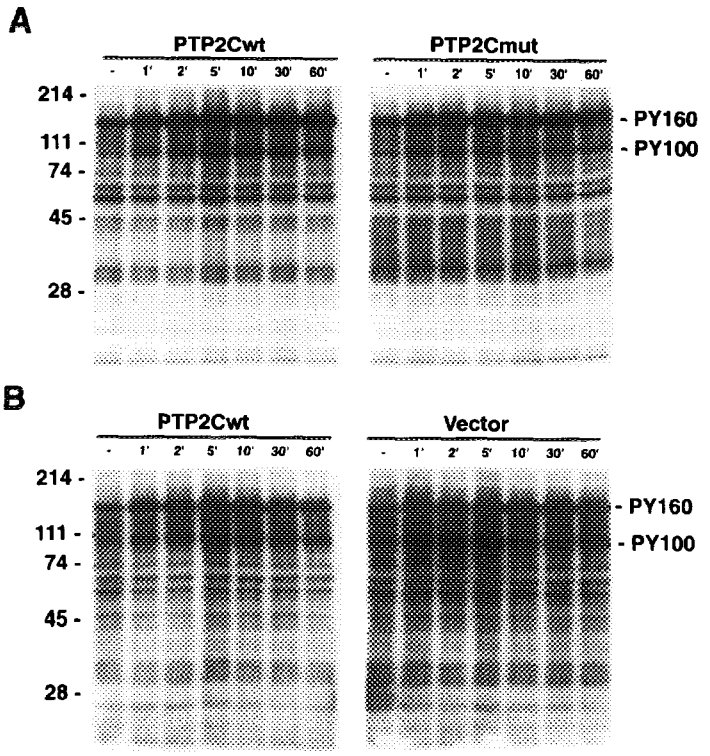

Figure 2. Insulin-elicited protein tyrosine phosphorylation in 293 cell lines. The 293 cell lines described in the text (PTP2Cwt, PTP2Cmut and vector) were exposed to 1 μM insulin for the specified times (1 to 60 min) or left in the basal state (-). Samples of the cell lysates (0.5% of a 10 cm plate) were immunoblotted for Ptyr.

Zhao et al. that the relative amounts of PTP2C in the PTP2Cwt and mut lines were about seven times that in the vector line. Zhao et al. have also shown that the relative activities of PTP2C in the vector, PTP2Cwt, and PTP2Cmut cell lines, as determined by assay of immunoprecipitated PTP2C, were 1:7:1, respectively.

The patterns of basal and insulin-elicited protein tyrosine phosphorylation in the three cell lines were examined by immunoblotting for Ptyr (Fig. 2). With the exception of a broad band at 35 to 45 kD in the PTP2Cmut line (see below), the pattern and intensity of tyrosine phosphorylation in the basal state were the same for the three lines (compare the lanes marked - in Fig. 2). Upon treatment of the cells with 1 μM insulin for various times, there was a rapid increase in tyrosine phosphorylation of polypeptides at 160 and 100 kD that persisted for at least an hour. The rate and the extent of tyrosine phosphorylation of these two polypeptides were almost identical in the three cell lines. The extent of increase in tyrosine phosphorylation of the 160 kD and 100 kD polypeptides was approximately 4-fold, as assessed by comparing the signal intensities from basal cells with those given by lower loads of the insulin-treated cells (data not shown).

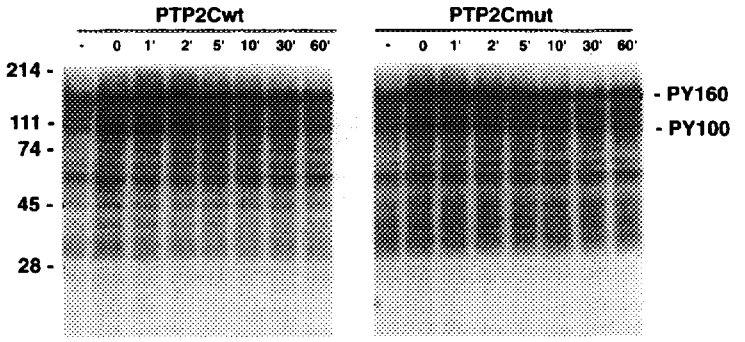

Figure 3. Tyrosine dephosphorylation upon withdrawal of insulin from 293 cell lines. The PTP2Cwt and PTP2Cmut cell lines were untreated (-), were treated with 1 μM insulin for 5 minutes (0), or were treated in this way, washed and incubated in insulin-free medium for the specified times (1 to 60 min). Samples of the cell lysates (0.5% of a 10 cm plate) were immunoblotted for Ptyr.

Although overexpression of the normal and mutant PTP2C did not alter the insulin-stimulated tyrosine phosphorylation, it seemed possible that the rate of dephosphorylation upon insulin withdrawal might be affected. Consequently the time courses of tyrosine dephosphorylation upon transfer of insulin-stimulated cells to an insulin-free medium were determined. In Fig. 3 the results with the PTP2Cwt and PTP2Cmut lines are compared. In both lines, the tyrosine phosphorylation of the 160 kD protein decreased to approximately the basal level in the period from 5 to 10 minutes after insulin withdrawal, whereas the decrease in the tyrosine phosphorylation of the 100 kD protein occurred slightly more rapidly, with it being evident in the period from 2 to 5 minutes after insulin withdrawal. The time courses for dephosphorylation of these two proteins in the vector cell line were similar to those in Fig. 3 (data not shown). Thus, the overexpression of normal or mutant PTP2C did not alter the rate at which insulin-stimulated tyrosine phosphorylation disappeared upon hormone removal.

Because IRS-1 migrates at 160 kD and because we detected it in lysates of 293 cells by immunoblotting (Fig. 4), our expectation was that the insulin-elicited 160 kD Ptyr protein was IRS-1. However, complete immunoprecipitation of IRS-1 from the lysates of the insulin-treated 293 cells did not deplete any of the 160 kD Ptyr protein (Fig. 4, compare the lanes designated IRS in parts A and B). Further evidence that the 160 Ptyr protein was not IRS-1 came from a comparison of the human 293 vector cells with mouse 3T3-L1 adipocytes. Immunoblotting for IRS-1 with antibodies against the carboxy terminal peptide of IRS-1, which is identical in human and murine IRS-1 (12), revealed that on a per mg protein basis, 293 cells contained only 1/15 the amount of IRS-1 as 3T3-L1 adipocytes, yet upon immunoblotting for Ptyr the signal from the protein at 160 kD in insulin-treated 293 cells was 1.5X that given by insulin-treated 3T-3L1 adipocytes (data not shown). The identity of the insulin-elicited 160 kD Ptyr protein in 293 cells thus remains to be determined. A strong possibility is that it is the

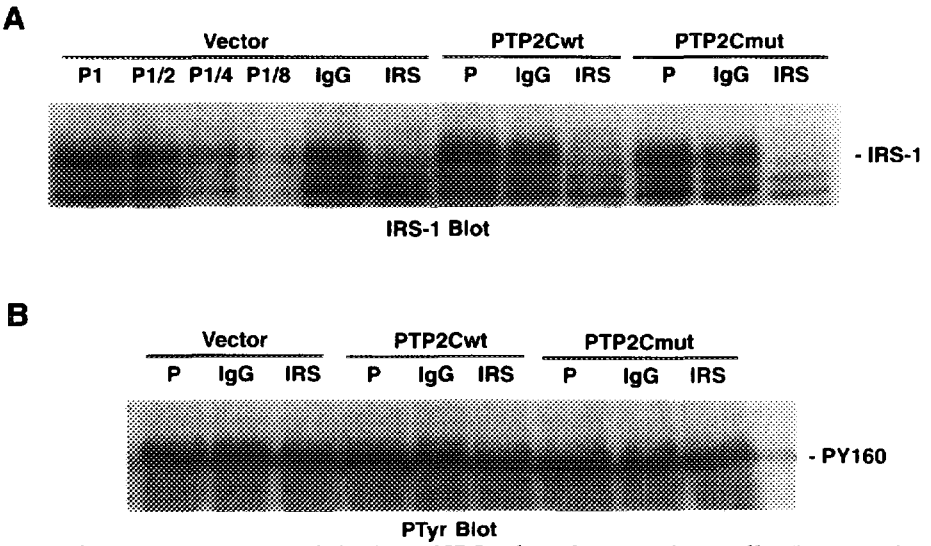

Figure 4. Immunoprecipitation of IRS-1 from lysates of 293 cells. Lysates of 293 cells that had been treated with 1 μM insulin for 5 min were immunoprecipitated with irrelevant antibodies or antibodies against IRS-1, as described in the Methods. Samples of the lysates prior to immunoprecipitation (P) and after immunoprecipitation with the irrelevant antibodies (IgG) or antibodies against IRS-1 (IRS) were immunoblotted for IRS-1 (Part A) and Ptyr (part B). In part A all lanes, except for vector P1/2, 1/4 and 1/8, contained 1% of the lysate from a 10 cm plate; these lanes contained 1/2, 1/4, and 1/8 this amount, respectively. In part B, all lanes contained 1% of the lysate from a 10-cm plate.

recently described insulin-elicited Ptyr protein designated IRS-2, which has approximately the same apparent size as IRS-1 (4,5).

The insulin-elicited Ptyr protein at 100 kD is the size of the β subunits of the insulin and insulin-like growth factor receptors. Most likely it is a mixture of both, since the 293 cell has been reported to have approximately 9,000 insulin receptors and 11,000 IGF I receptors (13). Our observation that maximal tyrosine phosphorylation of this protein in each cell line required 1 μM insulin (data not shown) supports this conclusion, since this high a concentration of insulin is required to saturate the IGF I receptor (14).

The one difference in protein tyrosine phosphorylation among the three cell lines is the occurrence of a broad band at 35 to 45 kD in the PTP2Cmut line, in both the basal and insulin-treated state (Fig. 2). Zhao et al. previously detected this hyperphosphorylated protein and described that it is membrane-associated and immunoprecipitates with the mutated PTP2C (9).

Discussion

If PTP2C were the phosphatase responsible for dephosphorylating the insulin-elicited 160 kD Ptyr protein and the insulin and IGF I receptors in vivo, then its

overexpression would be expected to result in lower extents of tyrosine phosphorylation of these proteins in response to insulin and a more rapid return to the basal levels of phosphorylation upon insulin withdrawal. The fact that these effects were not observed indicates that in vivo PTP2C does not have this function.

The results with the PTP2Cmut cell line support this conclusion. We have shown previously that the Ptyr form of recombinant IRS-1 is a good substrate for recombinant PTP2C and established that the basis of this effect is the binding of Ptyr sequences on IRS-1 to one or both SH2 domains of PTP2C (7). Other proteins that are multiply phosphorylated on tyrosine are also likely to be good substrates for the enzyme if this interaction occurs. The catalytically inactive point mutant of PTP2C, which can still associate with potential substrates via its functional SH2 domains, would be expected to compete with the endogenous PTP2C in binding to such good substrates. As a result, if PTP2C were responsible for dephosphorylation of either the 160 kD protein or the receptors in vivo, the PTP2Cmut line might have exhibited an increase in the extent of insulin-elicited tyrosine phosphorylation and a slower rate of dephosphorylation upon insulin withdrawal.

While this study was in progress, three reports appeared that describe similar results for two cell lines overexpressing the insulin receptor (15-17). In NIH 3T3/insulin receptor cells, expression of the PTP2C 459 Cys-to-Ser mutant had no effect on the tyrosine phosphorylation of IRS-1 or the insulin receptor in response to insulin. (15) In CHO/insulin receptor cells, overexpression of either PTP2C or of the 459 Cys-to-Ser mutant caused no change in the extent of IRS-1 tyrosine phosphorylation. Finally, in another study with CHO/insulin receptor cells, overexpression of PTP2C had no effect on the extent of tyrosine phosphorylation of IRS-1 or the insulin receptor, whereas overexpression of the 459 Cys-to-Ser mutant caused a very small increase in IRS-1 phosphorylation, which was not quantitated (17). None of these studies examined the rate of tyrosine dephosphorylation upon insulin withdrawal. In all three studies, it was found that overexpression of the catalytically inactive mutant PTP2C inhibited activation of the mitogen-activated protein kinase in response to insulin. Thus, on the basis of this finding it appears that catalytically active PTP2C is a positive effector for insulin signaling.

The tyrosine phosphatases that dephosphorylate IRS-1, IRS-2, and the insulin and IGF I receptors in vivo remain to be identified. A recent study presents evidence that the transmembrane phosphatase LAR is one of the phosphatases acting on the insulin receptor in vivo (18), and it is possible that it also acts on these other proteins. Since many of the Ptyr's on these proteins are complexed with SH2 domains, consideration should be given to the possibility that there exist tyrosine phosphatases that specifically act on a Ptyr-containing sequence complexed with an SH2 domain.

Finally, a surprising result of this study is the finding that the predominant 160 kD insulin-elicited Ptyr protein in 293 cells is not IRS-1. In the future, it will be important to determine whether this protein is IRS-2 or is, instead, an as yet uncharacterized substrate for the insulin/IGF I receptors.

Acknowledgments

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References

1. White, M.F. and Kahn, C.R. (1994) J. Biol. Chem. 269, 1-4.
2. Keller, S.R. and Lienhard, G.E. (1994) Trends in Cell Biol. 4, 115-119.
3. Myers, M.G., Jr., Sun, X.J., Cheatham, B., Jachna, B.R., Glasheen, E.M., Backer, J.M. and White, M.F. (1993) Endocrinology 132, 1421-1430.
4. Araki, E., Lipes, M.A., Patti, M-E., Brüning, J.C., Haag, B. III, Johnson, R.S., and Kahn, C.R. (1994) Nature 372, 186-190.
5. Tobe, K., Tamemoto, H., Yamauchi, T., Aizawa, S., Yazaki, Y., and Kadowaki, T. (1995) J. Biol. Chem. 270, 5698-5701.
6. Kuhné, M.R., Pawson, T., Lienhard, G.E. and Feng, G-S. (1993) J. Biol. Chem. 268, 11479-11481.
7. Kuhné, M.R., Zhao, Z., Rowles, J., Lavan, B.E., Shen, S-H., Fischer, E.H. and Lienhard, G.E. (1994) J. Biol. Chem. 269, 15833-15837.
8. Staubs, P.A., Reichart, D.R., Saltiel, A.R., Milarski, K.L., Maegawa, H., Berhanu, P., Olefsky, J.M., and Seely, B.L. (1994) J. Biol. Chem. 269, 27186-27192.
9. Zhao, Z., Tan, Z., Wright, J.H., Diltz, C.D., Shen, S-H., Krebs, E.G., Fischer, E.H. (1995) J. Biol. Chem., in press.
10. Lamphere, L. and Lienhard, G.E. (1992) Endocrinology 131, 2196-2202.
11. Zhao, Z., Larocque, R., Ho, W-T., Fischer, E.H. and Shen, S-H. (1994) J. Biol. Chem. 269, 8780-8785.
12. Keller, S.R. and Lienhard, G.E. (1994) in Molecular Biology of Diabetes (B. Draznin and D. LeRoith, eds.), Vol. II, pp. 95-114. Humana Press, Totwa, NJ.
13. Beitner-Johnson, D. and LeRoith, D. (1995) J. Biol. Chem. 270, 5187-5190.
14. Adamo, M., Roberts, C.T., Jr., and LeRoith, D. (1992) BioFactors, 3, 151-157.
15. Milarski, K.L. and Saltiel, A.R. (1994) J. Biol. Chem. 269, 21239-21243.
16. Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M. (1994) Mol. Cell. Biol. (1994) 6674-6682.
17. Yamauchi, K., Milarski, K.L., Saltiel, A.R. and Pessin, J.E. (1995) Proc. Natl. Acad. Sci. USA 92, 664-668.
18. Kulas, D.T., Zhang, W-R., Goldstein, B.J., Furlanetto, R.W. and Mooney, R.A. (1995) J. Biol. Chem. 270, 2435-2438.