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Multiple receptor-like protein tyrosine phosphatases in the form of chondroitin sulfate proteoglycan

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Abstract The possibility that some of the brain proteoglycans are receptor-like protein tyrosine phosphatases (PTPases) was investigated. Membrane-bound proteoglycan fractions were prepared from the postnuclear membrane fraction of 8-day-old rat brain by DEAE ion-exchange chromatography and CsCl density gradient centrifugation. The isolated proteoglycan fractions showed high PTPase specific activities together with the typical PTPase characteristics. Renaturation experiments indicated that chondroitin sulfate proteoglycans with 380- and 170-kDa core proteins carried the PTPase activity. The proteoglycan with 380-kDa core protein was identified as RPTPβ/ζ bearing HNK-1 carbohydrate.

Key words: Protein tyrosine phosphatase; Proteoglycan; HNK-1 carbohydrate; Rat brain

1. Introduction

Diverse cellular processes are regulated by protein tyrosine phosphorylation, the level of which is determined by the balance of protein tyrosine kinase and protein tyrosine phosphatase (PTPase) activities [1,2]. Recently, many types of receptor-like PTPases have been cloned and characterized [1–3]. Although ligands of the receptor-like PTPases are unknown, recent studies have suggested that some types of receptor-like PTPases are directly involved in cell–cell adhesion, and that the activities of PTPases are regulated by cell–substrate adhesion [4–6].

On the other hand, proteoglycans are now thought to play important roles in the regulation of cell adhesion, growth, motility and differentiation [7]. Proteoglycans are a family of proteins bearing sulfated glycosaminoglycans, which bind many extracellular matrix components and growth factors through their core protein and glycosaminoglycan portions [7].

Previously, we identified a phosphate-buffered saline-soluble chondroitin sulfate proteoglycan with a 300-kDa core protein (6B4 proteoglycan) using a monoclonal antibody (MAb6B4) [8]. We cloned cDNAs encoding this proteoglycan from a λgt11 rat whole brain cDNA library. Sequence analysis of the isolated cDNA clones indicated that 6B4 proteoglycan is highly homologous to a human receptor-like PTPase, RPTPβ/ζ [3,9]. Simultaneously, Maurel et al. [10] reported a cDNA encoding a chondroitin sulfate proteoglycan, phosphacan, from rat brain, and it became clear that 6B4 proteoglycan is identical to phosphacan. Phosphacan is considered from its cDNA structure to be an alternatively spliced extracellular variant of RPTPβ/ζ. Therefore, we attempted to identify proteoglycan-type PTPases in the rat brain, where many types of proteoglycans and PTPases are known to be present [11,12]. Here, we provide the first evidence that early postnatal rat brain indeed contains multiple proteoglycan-type PTPases, one of which is RPTPβ/ζ.

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Abbreviations: PTPase, protein tyrosine phosphatase; MAb, monoclonal antibody; DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride; *p*NPP, *p*-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

2. Materials and methods

2.1. Partial purification of proteoglycan-type PTPases

Thirty grams of whole brain tissue from 8-day-old Sprague–Dawley rats was homogenized in 8 volumes of 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 1 mM benzamidine and 50 mM Tris-HCl, pH 7.4 (buffer A) containing 0.32 M sucrose with a glass-Teflon Potter homogenizer. The homogenate was centrifuged at 1,000 × *g* for 5 min at 2°C and the resultant precipitate was washed under the same conditions. Combined supernatants were centrifuged at 105,000 × *g* for 60 min at 2°C to precipitate the postnuclear fraction. After washing once with 100 ml of 0.1 M sucrose/0.2 M NaCl/buffer A, the membrane proteins were solubilized in 200 ml of 1% CHAPS/0.1 M NaCl/buffer A, and insoluble material was removed by centrifugation at 20,000 × *g* for 60 min at 2°C. The supernatant was applied to a DEAE-Toyopearl column (2.5 × 5 cm; Tosoh Corporation) equilibrated with 0.5% CHAPS, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM benzamidine, 50 mM Tris-HCl, pH 7.4 (buffer B) containing 0.1 M NaCl. The column was washed with 0.25 M NaCl/buffer B, and then the proteins were eluted with 0.6 M NaCl/buffer B. After adjusting the density to 1.43 g/ml with solid CsCl, the eluate was centrifuged at 77,000 rpm at 4°C for 35 h using a Beckman TLA100.4 rotor. After centrifugation, the samples were collected in 10 fractions.

2.2. Preparation of substrate and assay of PTPase activity

Tyrosine phosphorylation of Raytide (Oncogene Science) was carried out according to the method described by Guan et al. [13]. Dephosphorylation of [^{32}P]phosphotyrosine-labeled Raytide (PTPase assay) was measured as described by Krueger et al. [3]. Hydrolysis of *p*-nitrophenyl phosphate (*p*NPPase assay) was carried out as described by Guan et al. [13]. One unit is defined as the activity which releases 1 nmol of phosphate per min.

2.3. Isolation of phosphacan/6B4 proteoglycan and RPTPβ/ζ cDNA clones

A λgt11 cDNA library was constructed with poly(A)$^+$ RNA from the whole brain of 18-day-old Sprague–Dawley rats as a template using random primers. Positive clones were isolated by screening this cDNA library with rabbit antiserum to the purified 6B4 proteoglycan and an adult Sprague–Dawley rat brain λgt11 cDNA library (Clonetech, USA) with probes prepared from the previously isolated clones. The clones thus isolated were as follows (cDNAs are common or belong to RPTPβ/ζ unless otherwise mentioned [9,10]): λrPG-9 (carrying nucleotide 1,639–2,294), λrPG-10 (2,200–4,520), λrPG-11 (2,097–3,774), λrPG-22

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(1,526–6,274; dvRPTPβ/ζ), λrPG-31 (2,427–5,715; phosphacan), λrPG-51 (-115–1,291), λrPG-61 (462–2,257), λrPG-222 (1,964–7,746; dvRPTPβ/ζ) and λrPG-302 (1,526–6,050). Nucleotide residues are numbered beginning with the first residue of the initiative methionine.

2.4. Preparation of antiserum against the C-terminal portion of phosphacan/6B4 proteoglycan (antiserum 31–5)

To adjust the reading frame and to create appropriate cloning sites, the AccI (4,183)/*Eco*RI (5,034) cDNA fragment from λrPG-31 was once subcloned in the pBSII ks(+) vector and then inserted into pGEX4T-2 (Pharmacia) using *Xho*I/*Not*I sites. The resultant construct (pGEX31–5) expressed the C-terminal portion of phosphacan/6B4 proteoglycan (amino acid residues 1,360–1,616) as a fusion protein with glutathione *S*-transferase (GST) in DH5α cells. The cDNA derived portion was prepared from the fusion protein with Pharmacia GST purification module and used to immunize New Zealand white rabbits.

2.5. Other methods

Protein concentration was determined using a BioRad protein assay kit with bovine serum albumin as a standard. Enzyme activity was recovered from the gel using the procedure described by Hager and Burgess [14], in that renaturation of proteins was performed for 15 h after dilution of guanidium chloride. Purification of phosphate-buffered saline-soluble proteoglycan was performed as described previously [15]. Digestion of proteoglycans with protease-free chondroitinase ABC (Seikagaku Corporation) was carried out as described previously except that *N*-ethylmaleimide was omitted [8].

3. Results

3.1. Partial purification and characterization of proteoglycan-type PTPases

For the purification of membrane-bound proteoglycans, the postnuclear fraction prepared from 8-day-old rat whole brains was solubilized with 1% CHAPS. When the solubilized material was applied to a DEAE-Toyopearl column, most of the PTPase activity was eluted from the column by 0.25 M NaCl. However, ~8% of the total activity remained in the column, and was eluted by 0.6 M NaCl. This highly negatively charged material was fractionated by CsCl density gradient centrifugation, which is often used to separate proteoglycans from normal protein. As shown in Fig. 1, most of the PTPase activity was recovered from the middle portion of the gradient (ρ = 1.35–1.55 g/ml), while nearly all of the protein remained on the top.

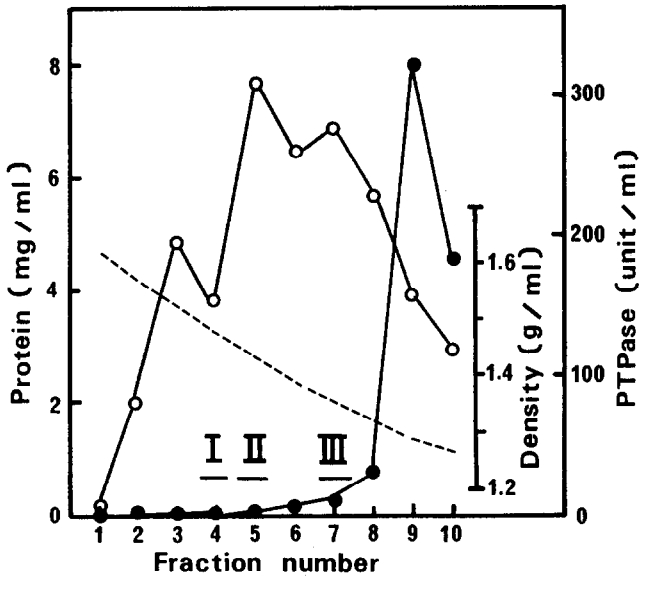

Fig. 1. CsCl density gradient centrifugation of proteins eluted from a DEAE-Toyopearl column. CHAPS-extract of the postnuclear fraction from rat brain was applied to a DEAE-Toyopearl column, and proteins were eluted with 0.6 M NaCl after washing with 0.25 M NaCl. The eluted proteins were fractionated by CsCl density gradient centrifugation. After centrifugation, the sample was fractionated into 10 tubes (500 μl/tube), and protein concentration (●), density (---), and PTPase activity (○) of each fraction were measured. The fractions designated I, II and III were analyzed further.

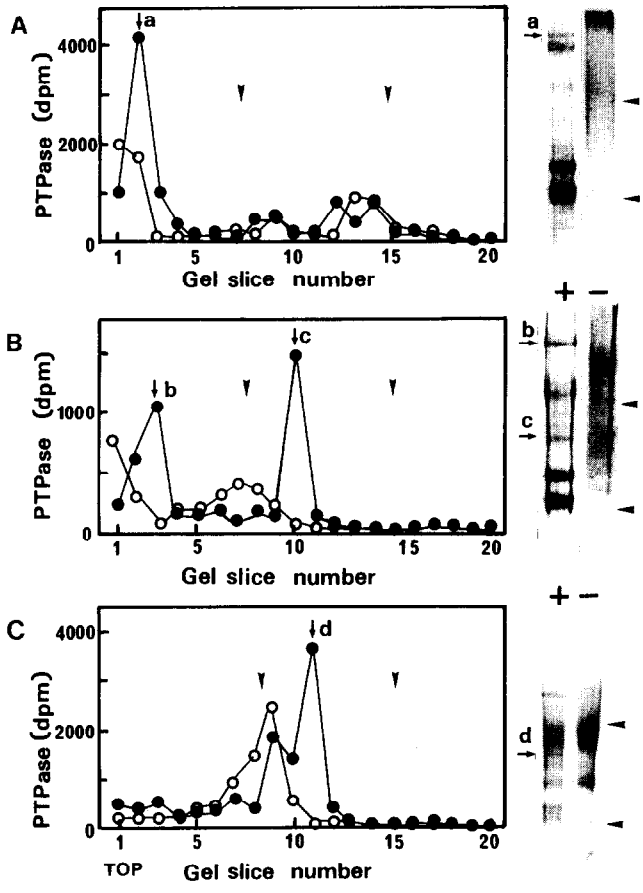

Fig. 2. Renaturation of PTPase activity after SDS-PAGE of fractions I, II and III. Aliquots of fractions I(A), II(B) and III(C) were digested with protease-free chondroitinase ABC (+) or incubated under the same conditions without the enzyme (−), and then applied to 5% SDS-PAGE. Left panels: After electrophoresis, the gel was cut into 2-mm slices, from which proteins were eluted and renatured. PTPase activity was measured using [^{32}P]phosphotyrosine-labeled Raytide; chondroitinase ABC-treated samples (●), intact samples (○). Right panels: Aliquots of the chondroitinase ABC-treated (+) or untreated (−) samples were electrophoresed on the same gel that used for renaturation, and the proteins were stained with Coomassie brilliant blue. Arrows indicate the positions of peaks of PTPase activity. Arrowheads indicate the positions of molecular weight markers of 200 kDa and 116 kDa (myosin and β-galactosidase, respectively).

To characterize this phosphatase activity, three fractions (I, II and III) from CsCl density gradient centrifugation were selected. Fig. 2 shows the results of SDS-PAGE analysis of these fractions. Before chondroitinase ABC digestion, the fractions yielded a very diffuse smear on electrophoresis (M_r > 1.0×10^5). However, discrete protein bands were observed in place of the smear after digestion (Fig. 2, right panels). In fraction I, 380-, 300-, 140- and 125-kDa bands were detected

as the major species, and 380-, 230-, 170-, 140-, and 125-kDa bands in fraction II. In fraction III, many core protein bands appeared after chondroitinase ABC digestion.

3.2. Properties of the PTPases in fractions I-III

The PTPase activity in these fractions were strongly inhibited by well-known PTPase inhibitors, vanadate, molybdate, zinc ion, and poly(Glu,Tyr). On the other hand, NaF and okadaic acid exerted no effects (data not shown). All known PTPases show optimal activity in the acidic range with *p*NPP as a substrate, and at neutral pH with peptide substrates [16]. This was also true for fractions I-III: Optimal activities of fractions I, II, and III were at pH 5.8, 6.3 and 5.8 with *p*NPP, respectively; pH 7.4 for all three fractions with [^{32}P]Raytide. Km values of these fractions for *p*NPP were all 5 mM, and those for [^{32}P]Raytide were 20 μM. All these properties of the phosphatases in fractions I-III were comparable to those of other PTPases reported previously [16]. Specific activities of fractions I, II and III for ^{32}P-Raytide were 2,060, 2,810 and 1,300 nmol/min/mg, respectively.

3.3. Assignment of PTPase activities to specific proteoglycan core proteins

From the high specific activity in each fraction, it was expected that the protein bands carrying the PTPase activities must be detectable as visible bands when separated on a gel. Accordingly, we next performed renaturation of the PTPases after SDS-PAGE of fractions I-III. When intact proteins were electrophoresed, PTPase activity was detected in two broad peaks (Fig. 2, left panels); one near the top of the gel and the other with M_r around 200 kDa. After chondroitinase ABC digestion, the former peak shifted to a position corresponding to 380-kDa into a single core protein band (Fig. 2A, a and Fig. 2B, b), and the latter peak at around 200 kDa shifted to 170 kDa (Fig. 2B, c and Fig. 2C, d). It was possible to assign the enzymatic activities to the specific core protein bands in fractions I and II, but not in fraction III because of a few core protein bands around 170 kDa.

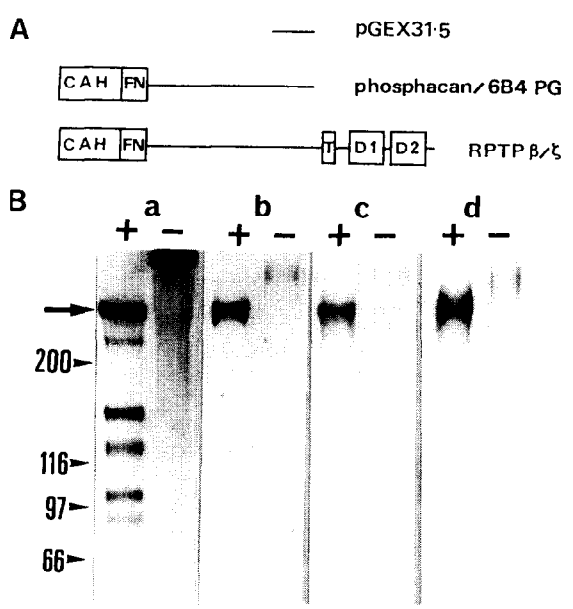

Fig. 3. Detection of phosphacan/6B4 proteoglycan in the soluble proteoglycan fraction with antibodies. (A) Schematic diagrams of phosphacan/6B4 proteoglycan and RPTPβ/ζ. Both forms contain carbonic anhydrase-like domain (CAH) and fibronectin type III repeat (FN). In addition, RPTPβ/ζ contains a transmembrane domain (T) and two phosphatase domains (D1 and D2). Antiserum 31–5 was raised against the C-terminal region of phosphacan/6B4 proteoglycan indicated by a bar (pGEX31–5). (B) Phosphate-buffered saline-soluble proteoglycan fraction prepared from 20-day-old rat whole brain was applied to 5% SDS-PAGE before (−) or after (+) chondroitinase ABC digestion. The proteins were stained with Coomassie Brilliant Blue (a), or analyzed by Western blotting using antiserum 31–5 (b), polyclonal antiserum against purified 6B4 proteoglycan (c), or MAb 6B4 (d). These antibodies reacted only with the phosphacan/6B4 proteoglycan (arrow).

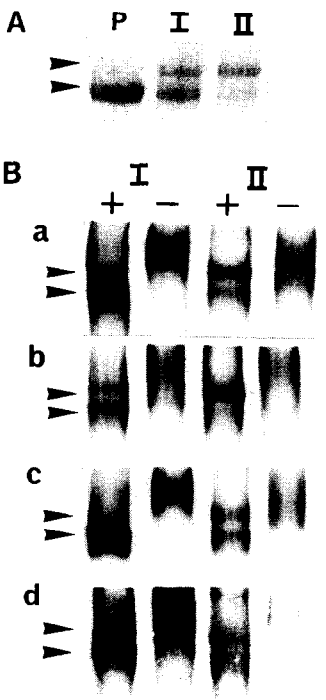

Fig. 4. Identification of RPTPβ/ζ and phosphacan/6B4 proteoglycan in fractions I and II. (A) Purified phosphacan/6B4 proteoglycan (P), and aliquots of fraction I (I) and fraction II (II) were digested with protease-free chondroitinase ABC. The samples were applied to 5% SDS-PAGE, and the proteins were stained with Coomassie Brilliant Blue. (B) Aliquots of fractions I (I) and II (II) were applied to 5% SDS-PAGE before (−) or after (+) chondroitinase ABC digestion. After electrophoresis, the samples were analyzed by Western blotting using polyclonal antiserum against purified 6B4 proteoglycan (a), MAb 6B4 (b), antiserum 31–5 (c) or HNK-1 monoclonal antibody (d). The arrowheads indicate the positions of 380 kDa and 300 kDa species.

3.4. Identification of the 380-kDa core protein band as RPTPβ/ζ

Phosphacan is considered to be an alternatively spliced extracellular variant of RPTPβ/ζ (Fig. 3A). We prepared antiserum 31–5 against the C-terminal portion of phosphacan/6B4 proteoglycan. Not only the antiserum against the purified 6B4 proteoglycan and MAb 6B4 but also antiserum 31–5 recognized the purified 6B4 proteoglycan (data not shown). Among the various soluble proteoglycans, the three antibodies specifically recognized a 300-kDa core protein of 6B4 proteoglycan (Fig. 3B). Electorophoretic mobility of the core protein of purified 6B4 proteoglycan was identical with that of the 300-kDa core protein species in fractions I and II (Fig. 4A).

We next examined the possibility that some of the proteoglycan-type PTPases may react with antibodies against 6B4

proteoglycan. Western blotting of fractions I and II indicated that both polyclonal and monoclonal antibodies against 6B4 proteoglycan reacted with 380- and 300-kDa core protein species (Fig. 4B, a and b). Antiserum 31–5 also reacted with the 380- and 300-kDa core proteins in fractions I and II (Fig. 4B, c). The 300-kDa core protein seems to be phosphacan/6B4 proteoglycan recovered in the membrane fraction probably because of the specific binding to receptors on the plasma membrane. No proteoglycan with a 380-kDa core protein was found in the soluble proteoglycan fraction. These results clearly indicate that the PTPase with 380-kDa core protein is RPTPβ/ζ. The three kinds of antibodies did not react with 170-kDa core protein species (data not shown), suggesting that the PTPase with a 170-kDa core protein is not highly structurally related to RPTPβ/ζ.

4. Discussion

We demonstrated that there indeed exist multiple proteoglycan-type PTPases in the rat brain. RPTPβ/ζ was identified here as a membrane-bound proteoglycan with a 380-kDa core protein. It has also been shown very recently that RPTPβ/ζ can be expressed in the form of proteoglycan by human 293 cells transfected with an expression plasmid encoding the full-length RPTPβ/ζ [17]. Besides RPTPβ/ζ, several types of PTPases have clusters of possible sequences for glycosaminoglycan attachment sites (Ser-Gly or Gly-Ser). RPTPγ is typical of these, and it is possible that the proteoglycan-type PTPase with a 170-kDa core protein is RPTPγ [18].

Proteoglycans play crucial roles in cell adhesion, motility, growth and differentiation through the process of binding to various extracellular matrix molecules and growth factors [7]. On the other hand, the level of protein tyrosine phosphorylation is regulated by cellular interactions with these molecules [1,2,6]. Our findings that some membrane-bound PTPases are proteoglycans would explain the functional overlap between the two classes of molecules.

Modification of PTPases with glycosaminoglycans should be able to highly diversify the functional aspects of these molecules in cell–cell and cell–substratum interactions. Precise glycosylation patterns can be determined only from proteoglycans purified from tissue. This study, for the first time, succeeded in the partial purification of proteoglycan-type PTPases from the brain. Phosphacan/6B4 proteoglycan carries HNK-1 carbohydrates in addition to chondroitin sulfate [8,19]. The finding that RPTPβ/ζ is also substituted with HNK-1 carbohydrate (Fig. 4B, d) suggests that post-translational modification of RPTPβ/ζ is similar to that of phosphacan/6B4 proteoglycan. Future studies are required to determine the ligand molecules, target proteins, and the regulatory mechanisms of the proteoglycan-type PTPases.

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References

[1] Carbonnearu, H. and Tonks, N.K. (1992) Annu. Rev. Cell Biol. 8, 463–493.
[2] Walton, K.M. and Dixon, J.E. (1993) Annu. Rev. Biochem. 62, 101–120.
[3] Krueger, N.X. and Saito, H. (1992) Proc. Natl. Acad. Sci. USA 89, 7417–7421.
[4] Gebbink, M.F.B.G., Zondag, G.C.M., Wubbolts, R.W.W., Beijersbergen, R.L., Etten, I. and Moolenaaar, W.H. (1993) J. Biol. Chem. 268, 16101–16104.
[5] Brady-Kalnay, S.M., Flint, A.J. and Tonks, N.K. (1993) J. Cell Biol. 122, 961–972.
[6] Maher, P.A. (1993) Proc. Natl. Acad. Sci. USA 90, 11177–11181.
[7] Ruoslahti, E. (1989) J. Biol. Chem. 264, 13369–13372.
[8] Maeda, N., Matsui, F. and Oohira, A. (1992) Dev. Biol. 151, 564–574.
[9] Levy, J.B., Canoll, P.D., Silvennoinen, O., Barnea, G., Morse, B., Honegger, A.M., Huang, J.-T., Cannizzaro, L.A., Park, S.-H., Druck, T., Huebner, K., Sap, J., Ehrlich, M., Musacchio, J.M. and Schlessinger, J. (1993) J. Biol. Chem. 268, 10573–10581.
[10] Maurel, P., Rauch, U., Flad, M., Margolis, R.K. and Margolis, R.U. (1994) Proc. Natl. Acad. Sci. USA 91, 2512–2516.
[11] Herndon, M.E. and Lander, A.D. (1990) Neuron 4, 949–981.
[12] Sahin, M. and Hockfield, S.J. (1993) J. Neurosci. 13, 4968- 4978.
[13] Guan, K., Broyles, S.S. and Dixon, J.E. (1991) Nature 350, 359–362.
[14] Hager, D.A. and Burgess, R.R. (1980) Anal. Biochem. 109, 76- 86.
[15] Oohira, A., Matsui, F., Matsuda, M., Takida, Y. and Kuboki, Y. (1988) J. Biol. Chem. 263, 10240–10248.
[16] Tonks, N.K., Diltz, C.D. and Fischer, E.H. (1988) J. Biol. Chem. 263, 6731–6737.
[17] Barnea, G., Grumet, M., Milev, P., Silvennoinen, O., Levy, J.B., Sap, J. and Schlessinger, J. (1994) J. Biol. Chem. 269, 14349–14352.
[18] Barnea, G., Sivennoinen, O., Shaanan, B., Honegger, A.M., Canoll, P.D., D'eustachio, P., Morse, B., Levy, J.B., Laforgia, S., Huebner, K., Musacchio, J.M., Sap, J. and Schlessinger, J. (1994) Mol. Cell Biol. 13, 1497–1506.
[19] Rauch. U., Gao, P., Janetzko, A., Flaccus, A., Hilgenberg, L., Tekotte, H., Margolis, R.K. and Margolis, R.U. (1991) J. Biol. Chem. 266, 14785–14801.