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Purification of the platelet-derived growth factor receptor by using an anti-phosphotyrosine antibody

(anti-phosphotyrosine/immunopurification/soluble receptor assay)

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ABSTRACT **The platelet-derived growth factor (PDGF) receptor is a 180-kDa membrane glycoprotein. A protein of identical size, lectin affinity, and isoelectric point has been identified as a major substrate for PDGF-activated tyrosine kinase in stimulated 3T3 cells. We have purified this tyrosine-phosphorylated protein to homogeneity by using anti-phosphotyrosine immunoaffinity and lectin affinity steps. Demonstration that this purified tyrosine phosphoprotein is the PDGF receptor necessitated development of an assay capable of identifying specific ^{125}I-labeled PDGF binding activity in soluble receptor preparations. PDGF receptor solubilized from 3T3 cell membranes with the detergent octyl β-D-glucoside was precipitated on an artificial liposome matrix after receptor aggregation with concanavalin A. Precipitated binding sites display affinity and kinetic characteristics of PDGF receptors in cells and membranes. Preparations of the 180-kDa phosphoprotein that are >90% homogeneous by silver stain and by [^{35}S]methionine protein autoradiography have specific high affinity ^{125}I-labeled PDGF binding sites (equilibrium dissociation constant, 0.1 × 10^{-9} M). Binding activity enrichment in this preparation reflects an 11,000-fold purification of binding activity in intact cells. These data demonstrate that the 180-kDa substrate of the PDGF-stimulated tyrosine kinase is the PDGF receptor. Furthermore, these methods provide a means of purifying this and other tyrosine kinase substrates from growth factor-stimulated cells.**

Platelet-derived growth factor (PDGF) and its structural analogue, the product of the v-*sis* oncogene, bind to a specific cell-surface receptor and thereby initiate the sequence of intracellular events leading to DNA replication (1–5). The PDGF receptor has been characterized by radioligand binding and cross-linking studies as a 180-kDa plasma membrane glycoprotein with high affinity and specificity for PDGF (6, 7). Like the receptors for epidermal growth factor (EGF), insulin, and somatomedin C, the PDGF receptor is closely coupled to tyrosine kinase activity, which is maximally stimulated when the receptor is occupied (8–13). One of the main substrates of PDGF-activated tyrosine kinase is a 180-kDa membrane glycoprotein that has the size, lectin affinity pattern, and isoelectric point of the PDGF receptor, suggesting that the binding and substrate molecules are identical (12, 13).

Further characterization of the structure and function of the PDGF receptor requires recovery of purified receptor in an active form. However, PDGF receptor purification has been impeded by several obstacles: (*i*) Commonly applied assays for soluble binding activity using differential precipitation or gel filtration are ineffective in separating bound from free ligand (unpublished observations). (*ii*) Rich tissue sources of PDGF receptor have not been identified and tissue binding sites may be obscured by tightly bound PDGF released from platelets during tissue preparation (7). (*iii*) Anti-receptor antibodies, such as those that have been used for studies of insulin, EGF, and transferrin receptors, have not been available for the PDGF receptor.

Recent studies demonstrate that anti-phosphotyrosine antibodies select from extracts of PDGF-stimulated cells a 180-kDa phosphoprotein with several physical characteristics expected of the PDGF receptor (12). We now report the purification of this protein to homogeneity and copurification of high-affinity PDGF binding activity. Assessment of binding activity in purified preparations was possible with an assay that uses detergent dilution and acetone precipitation of soluble PDGF binding sites on artificial liposomes (14, 15). The precipitation was facilitated by incubating the receptor preparation with concanavalin A, a lectin that binds to PDGF receptor but does not interfere with the binding of PDGF to the receptor sites.

MATERIALS AND METHODS

Reagents. Electrophoretically homogeneous PDGF was purified from outdated human platelets and iodinated by the Iodo-gen method as described (4). Phosphatidylcholine was obtained from Avanti Biochemicals, (Birmingham, AL), and liposomes were prepared as described (14). Octyl β-D-glucoside was obtained from Calbiochem–Behring, *N*,*N*′,*N*″-triacetylchitotriose was from Sigma, calf serum was from Colorado Serum (Denver, CO), and [^{35}S]methionine and [^{32}P]-orthophosphoric acid were from New England Nuclear. Platelet-poor plasma was prepared as described previously. Wheat germ agglutinin (Sigma) was coupled to CNBr-Sepharose (Pharmacia) at 20 mg of protein per g of Sepharose. Silver stain kit was from Accurate Chemicals (Westbury, NY). Molecular weight standards were from Bio-Rad.

Membrane Preparation and Solubilization. Membranes were prepared from BALB/c 3T3 cells and stored at −70°C as described (7). Thawed membranes were pelleted by centrifugation at 39,000 × *g* for 20 min at 4°C and were resuspended in cold solubilization buffer (0.125 M Tris maleate, pH 6.0/0.16 M NaCl/2 mM $CaCl_2$/1 mM phenylmethylsulfonyl fluoride) at a concentration of 10–20 mg/ml. Membrane suspensions were then sonicated on ice with a Heat System Ultrasonics model LW-225R sonicator set at 60% pulse setting for 12 pulses, and an aliquot of this suspension was assayed for presolubilization membrane binding. Membrane proteins were solubilized by addition of 0.1 vol of stock 0.6 M octyl β-D-glucoside, or as described in the figure legends, and were incubated for 20 min on ice. Insoluble material was removed by centrifugation as noted.

Soluble Receptor Assay. Solubilized receptor preparations, from solubilized membranes or from wheat germ agglutinin

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Abbreviations: PDGF, platelet-derived growth factor; ^{125}I-PDGF, ^{125}I-labeled PDGF; WGA, wheat germ agglutinin.

(WGA)-Sepharose eluates (typically 1 vol = 250 μl), were mixed with 3.2 vol of phosphatidylcholine liposomes (2 mg/ml) and 1.1 vol of Con A (1 mg/ml). The mixture was then diluted with 2 vol of 20 mM Tris maleate, pH 6.0/2 mM $CaCl_2$ and 5 vol of Tris/NaCl to yield final concentrations of 25 mM Tris and 0.4 M NaCl. Chilled acetone (6 vol) was added over 15 sec during Vortex mixing. Liposomes were recovered by centrifugation at 16,000 × *g* for 20 min at 4°C, and the pellet was resuspended in 150–250 μl of 60 mM NaCl/25 mM Tris·HCl, pH 7.4/2 mM $CaCl_2$ by repeated aspiration through a 22-gauge needle.

To the liposome suspension (30 μl) was added 570 μl of buffer containing 25 mM Tris·HCl, pH 7.4/60 mM NaCl/70 μl of platelet-poor plasma, and 10 μl of ^{125}I-labeled PDGF (^{125}I-PDGF) (10,000–30,000 cpm at 10,000 or 20,000 cpm/ng). This mixture was incubated at 25°C with gentle shaking for 3 hr and was then filtered and washed as described (7). Filters were counted in a Beckman Gamma 5500 γ counter. Specific binding was determined as the difference between means of triplicate assays in the absence and presence of 100-fold excess unlabeled PDGF.

Receptor Purification. BALB/c 3T3 cells (American Type Culture Collection CCL 163, passage 65–75) were grown in Dulbecco's modified Eagle's minimal essential medium (DME medium) (4.5 of glucose/liter) with 10% calf serum after seeding in roller bottles at 8 × 10^6 cells per 850-cm^2 roller bottle (Falcon). Medium was changed on day 2 and cells were processed on days 7–9. Each roller bottle was washed three times with 50 ml of DME medium, incubated, and rotated at 4°C in 20 ml of serum-free DME medium with or without 4.6 nM PDGF. Medium was removed by aspiration after 3 hr and cells were solubilized *in situ* by using glass beads with 20 ml per bottle of ice-cold Tris-buffered Triton X-100 (100 μM sodium metavanadate/50 mM NaF/30 mM sodium pyrophosphate/50 mM NaCl/5 mM EDTA/10 mM Tris·HCl, pH 7.4/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride) with bovine serum albumin (1 mg/ml). Lysates were centrifuged at 39,000 × *g* for 20 min at 4°C. Supernatants were dialyzed over 14 hr against four changes, 80 vol each, of Tris-buffered Triton X-100 without bovine serum albumin.

Dialyzed lysates were clarified by centrifugation, then loaded over 3 hr at 4°C on 10-ml (packed volume) anti-phosphotyrosine-Sepharose columns. Columns were then washed sequentially with Tris-buffered Triton X-100 without bovine serum albumin, and finally with Tris-buffered octyl-β-D-glucoside (identical to Tris-buffered Triton X-100 except 20 mM octyl β-D-glucoside is substituted for Triton X-100). Phosphotyrosine-containing proteins were eluted by using buffer containing 40 mM phenyl phosphate/3.3 mM Tris·HCl, pH 7.4/30 mM NaCl/20 mM octyl β-D-glucoside.

The eluates were incubated with WGA-Sepharose (100 μl of packed beads per ml of elutate) for 2–3 hr at 4°C on a rocker platform. WGA-Sepharose was washed three times with 0.1 M NH_4HCO_3/15 mM octyl β-D-glucoside before elution with the same buffer containing 3 mM *N,N',N''*-tria-

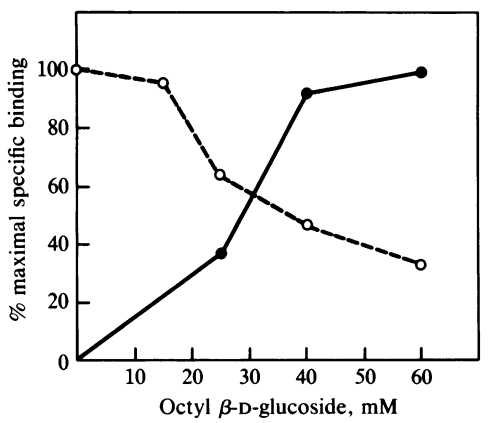

FIG. 1. Solubilization of PDGF receptors from 3T3 cell membranes. Membranes were treated with the indicated concentrations of octyl β-D-glucoside and were separated into soluble (●) and membrane (○) fractions by centrifugation at 100,000 × *g* for 90 min. Specific ^{125}I-PDGF binding was measured in the soluble fraction by the liposome precipitation assay without Con A. The residual membrane protein binding was measured as described (7). Maximal specific binding for the membranes (0 mM octyl β-D-glucoside) was 21,000 cpm per mg of membrane protein. Maximal specific binding for the soluble preparation (60 mM octyl β-D-glucoside) was 10,000 cpm per mg of protein.

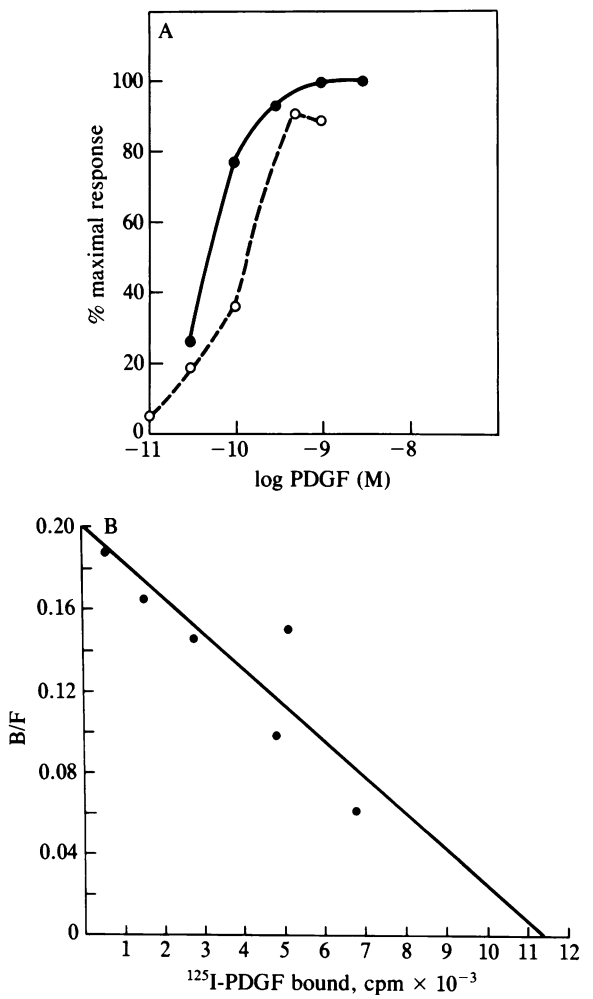

FIG. 2. Comparison of the ability of PDGF to stimulate mitogenesis and to compete for ^{125}I-PDGF binding sites precipitated from a soluble preparation. (*A*) Soluble extracts were prepared from 3T3 cell membranes and were precipitated on liposomes. The ability of PDGF at the indicated concentrations to inhibit the specific binding of ^{125}I-PDGF to precipitated extracts (●) and to stimulate thymidine uptake (○) was determined. Each point represents the mean of duplicate or triplicate determinations from two separate experiments. Maximal response refers to the total inhibition of specific binding and to the maximal stimulation of thymidine uptake, respectively. (*B*) Scatchard plot of ^{125}I-PDGF binding sites in precipitated extracts of 3T3 cell membranes. Cell membranes (30 mg of protein) were solubilized in 60 mM octyl β-D-glucoside. Approximately 50% of the membrane protein was effectively solubilized (150,000 × *g* for 1 hr) and was precipitated onto liposomes as described. ^{125}I-PDGF binding was measured on the precipitated vesicles over a range of ^{125}I-PDGF concentration and specific binding (B/F) was plotted. Soluble preparation contained 3 mg of protein per ml, and 30 μl of this preparation was precipitated for each determination in the binding assay.

cetylchitotriose. The amount of receptor protein was estimated by comparison of the silver stain intensity of the 180-kDa band on polyacrylamide gels to that of known amounts of standard proteins added at several dilutions.

RESULTS

Soluble Binding Assay. The nonionic detergent, octyl β-D-glucoside, extracted ^{125}I-PDGF binding sites from 3T3 cell membranes in a manner dependent on octyl β-D-glucoside concentration (Fig. 1). Recovery of extracted binding sites by precipitation of solubilized receptors on phosphatidylcholine vesicles ranged from 10%–40%, unless Con A was included before the precipitation. With the addition of Con A, binding site recovery was ≈3-fold higher. In control experiments performed in the absence of receptor preparation or using soluble receptor preparations from 3T3 cells that had lost PDGF receptors after cell transformation with the Abelson leukemia virus (unpublished data), there was no detectable specific ^{125}I-PDGF binding on the liposome precipitation assay.

The solubilized precipitated binding activity was further characterized by comparing the concentration of unlabeled PDGF required to inhibit ^{125}I-PDGF binding to that required for stimulation of [^{3}H]thymidine incorporation (Fig. 2*A*). The PDGF concentration required for half-maximal inhibition of ^{125}I-PDGF binding (0.04 nM) was comparable to that providing half-maximal thymidine uptake (0.1 nM). Reduced and alkylated PDGF neither inhibited ^{125}I-PDGF binding to precipitated receptor nor stimulated thymidine uptake at a concentration of 1 nM (data not shown). Epidermal growth factor, nerve growth factor, and fibroblast growth factor similarly failed to block ^{125}I-PDGF binding to receptor in the liposome assay. By Scatchard analysis, the equilibrium dissociation constant for the binding of PDGF to the precipitated sites was 0.03×10^{-9} M, and the density of sites was 200–300 fmol of receptor per mg of precipitated protein, assuming 1:1 ligand–receptor binding (Fig. 2*B*). As described for ^{125}I-PDGF binding to receptor sites in intact membranes (7), PDGF binding to liposome-precipitated receptor was not dissociable by addition of unlabeled PDGF or by dilution after a 30 min incubation at 37°C. However, addition of the polyanionic compound suramin (1 mM) effected rapid dissociation of >80% of specific binding (data not shown).

Receptor Purification. Phosphotyrosine proteins in lysates from unstimulated and PDGF-stimulated 3T3 cells were purified by anti-phosphotyrosine-Sepharose chromatography followed by lectin affinity chromatography using WGA-Sepharose (Fig. 3). The product 180-kDa phosphoprotein, was purified to >90% homogeneity as assessed by NaDodSO$_4$/polyacrylamide electrophoresis and silver staining for protein. A similar degree of purity was observed when the product was analyzed by autoradiography of [^{35}S]methionine-labeled proteins (Fig. 3). Only those lysates from PDGF-stimulated cells yielded the 180-kDa protein.

The purified preparation containing the 180-kDa phosphoprotein (from PDGF-stimulated cells) had a high level of specific ^{125}I-PDGF binding activity, whereas a comparable preparation from unstimulated cells did not bind a significant amount of ^{125}I-PDGF (Fig. 4). The binding affinity of the

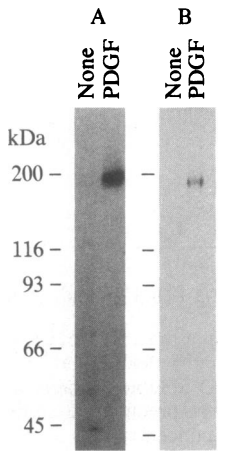

FIG. 3. Autoradiogram and silver stain of NaDodSO$_4$/polyacrylamide gels of purified preparations from unstimulated and PDGF-stimulated cells. (*A*) Parallel cultures of BALB/c 3T3 cells were preincubated with 200 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq) in methionine-free DME medium for 3 hr. Unstimulated (None) and PDGF-stimulated (PDGF) cells were processed, and lectin affinity eluates were subjected to NaDodSO$_4$/polyacrylamide electrophoresis on 7% gels and then to autoradiography for 10 hr with Kodak XR-5 film and Cronex enhancing screens. (*B*) Parallel unlabeled roller bottle cultures without (None) or with (PDGF) PDGF-stimulation were processed through the same immunoaffinity and lectin affinity steps as in *A*. Purified preparations from one roller bottle each were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, and the proteins were detected by silver stain.

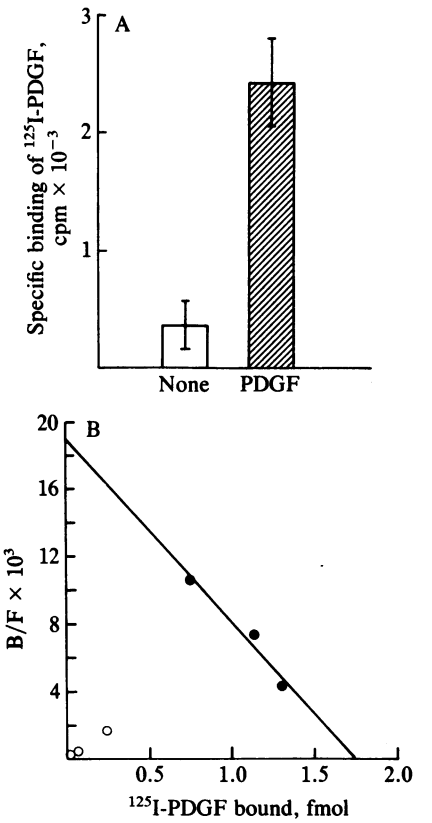

FIG. 4. PDGF binding characteristics of purified preparations from unstimulated and from PDGF-stimulated cells. (*A*) By using the soluble binding assay, purified preparations from unstimulated (None) cell lysates and from PDGF-stimulated (PDGF) cell lysates were precipitated on liposomes, incubated with ^{125}I-PDGF in the absence or presence of excess unlabeled PDGF, filtered, and filters were counted. Specific binding accounted for 55% of total binding in the stimulated preparation. Depicted is the specific binding activity present in purified preparations from 2.5×10^8 cells (one roller bottle). These data are representative of binding activity in two separate purification preparations. (*B*) Purified protein from unstimulated (○) and from PDGF-stimulated (●) cells was assayed for specific PDGF binding with three concentrations of ^{125}I-PDGF (10,000 cpm/ng). Scatchard analysis of precipitated binding sites yields an equilibrium dissociation constant of 0.1×10^{-9} and 6.5 fmol of ^{125}I-PDGF binding sites per 2.5×10^8 cells (each triplicate determination reflects binding from 0.27 roller bottles).

Table 1. Purification of the PDGF receptor from BALB/c 3T3 cells

	Protein recovered per 2.3×10^8 cells, μg	Total ^{125}I-PDGF high-affinity binding, pmol per fraction	Specific ^{125}I-PDGF binding activity, pmol per mg of protein	Purification factor, -fold
Intact cells		2.8 (100)	0.03	1
Soluble cell lysate	25,000	ND		
Immunoaffinity eluate	0.32	0.058 (2.1)	180	6,000
Lectin affinity eluate	0.02	0.0066 (0.23)	330	11,000

Specific ^{125}I-PDGF binding activity from intact cells was assayed by the filtration assay as described (7). Binding sites from immunoaffinity and lectin affinity eluates were measured by the lectin-facilitated precipitation assay. Binding activity was not detectable (ND) with this assay in crude cell lysates. Total recovery of the 180-kDa protein from lectin affinity eluates ranged from 20 to 100 ng per 2.3×10^8 cells (one roller bottle). Values in parentheses represent percentage of total ^{125}I-PDGF high-affinity binding relative to intact cells.

purified receptor was assessed by limited Scatchard analysis. The calculated equilibrium dissociation constant of 0.1×10^{-9} M (Fig. 4) was similar to that found for the receptor in intact cells and membranes (7). The maximum specific binding activity derived from the Scatchard plot was 325 pmol per mg of receptor protein. This value represents an 11,000-fold purification of the specific binding activity over that of intact cells (Table 1).

Theoretical univalent receptor–ligand binding for purified 180-kDa receptor protein should yield 5.6 nmol of ^{125}I-PDGF bound per mg of protein. Our recovery of less than theoretical binding activity reflects several factors. The measurement of binding requires precipitation of the receptor from dilute immunoaffinity eluates, a procedure that is only 20%–40% efficient, as assessed by precipitation of ^{32}P-labeled eluates. Thus, a substantial number of potential binding sites in these dilute solutions are not detected by our assay. The receptors that are precipitated on the vesicles may not retain full binding activity because of damage to the molecules that occurs during purification. No additional binding activity was recovered after suramin wash of WGA-Sepharose-bound receptor, suggesting that bound PDGF was not obscuring potential binding sites (data not shown).

Incubation of purified receptor preparations with [γ-^{32}P]ATP yielded incorporation of radiolabel into the receptor protein (data not shown). If this reflects anticipated additional autophosphorylation, label incorporation into this tyrosine-phosphorylated receptor was not stimulated by PDGF.

DISCUSSION

We have purified the PDGF receptor from PDGF-stimulated cells by using an anti-phosphotyrosine antibody in conjunction with lectin affinity chromatography. The identification of the purified protein as the receptor is based on the use of a lectin-facilitated precipitation assay. The purified 180-kDa protein has high affinity binding for ^{125}I-PDGF. A parallel preparation from identical unstimulated (by PDGF) cells lacked high affinity PDGF binding sites and did not contain the 180-kDa phosphoprotein. These data show that the 180-kDa substrate of PDGF-stimulated tyrosine kinase is the PDGF receptor. The molecular mass of the receptor purified by these methods agrees with the size of the PDGF binding sites labeled by cross-linking ^{125}I-PDGF to intact cells (2, 3) or membrane preparations (7). A protein of similar size is phosphorylated by PDGF stimulation of membrane preparations or soluble preparations in the presence of [γ-^{32}P]ATP (8, 11).

The receptor protein purified by the anti-phosphotyrosine antibody is in its "activated" tyrosine-phosphorylated form. The apparent affinity (equilibrium dissociation constant) of this form is comparable to the affinity of the native unstimulated receptor prior to purification (7). Thus, *in vivo* tyrosine phosphorylation apparently does not significantly alter the affinity of the receptor for ligand.

Although the overall yield of purified receptor is low, the methods used provide sufficient amounts of receptor for microsequencing and for use as antigen for antibody generation. The yield of receptor has improved during the preparation of this manuscript from the 20 ng per roller bottle figure cited in Table 1 to >100 ng of receptor per roller bottle. Availability of active preparations of purified receptor will allow studies to determine whether PDGF receptor shares enzymatic activities, such as tyrosine kinase and phosphatidylinositol kinase (16, 17), with other growth factor receptors and oncogene products.

The soluble assay used to measure ^{125}I-PDGF binding activity in purified receptor preparations is similar to assays previously developed for measuring ligand binding to the receptors for low density lipoprotein and nerve growth factor (14, 15). The efficiency of the assay was greatly enhanced by preincubating the soluble receptors with Con A. This assay should be useful in purification of PDGF receptor from tissue sources. Furthermore, the immunoaffinity purification step using Sepharose-coupled anti-phosphotyrosine antibody may have general utility in purifying other cellular substrates for growth factor-activated tyrosine kinase.

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