CHROMSYMP. 802

MICROSCALE STRUCTURE ANALYSIS OF A HIGH-MOLECULAR-WEIGHT, HYDROPHOBIC MEMBRANE GLYCOPROTEIN FRACTION WITH PLATELET-DERIVED GROWTH FACTOR-DEPENDENT KINASE ACTIVITY

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SUMMARY

General methods for the study of the primary structure of picomole quantities of large, hydrophobic membrane glycoproteins with blocked amino-termini have been developed. Three techniques designed to be used in concert with each other are described: first, modified protein preparation and fragmentation techniques; secondly, a simple but very selective two-dimensional reversed-phase high-performance liquid chromatography system for the resolution of complex mixtures of small to medium-sized tryptic peptides on Vydac C_4, C_{18} and diphenyl columns and thirdly, a two-dimensional separation method for large, denatured (CNBr) polypeptide fragments by size-exclusion high-performance liquid chromatography, combined with either reversed-phase high-performance liquid chromatography (C_4) or sodium dodecyl sulphate polyacrylamide gel electrophoresis in conjunction with electroblotting and autoradiography. These methods were applied to studies of the platelet-derived growth factor receptor. Starting with 500 pmoles of purified protein, a total of 232 amino acids were sequenced.

INTRODUCTION

The specific growth-promoting activity of platelet-derived growth factor (PDGF) upon its target cells (fibroblasts, glial cells, arterial smooth-muscle cells)[1] is mediated through interaction with a cell surface PDGF receptor[2]. The PDGF/PDGF receptor system is of great interest, since a structural relationship between this growth factor and viral (v-sis) and cellular (c-sis) oncogenes has been discovered[3,4]. The PDGF receptor is a monomeric (MW 185 000) intrinsic membrane glycoprotein[5] with PDGF-dependent tyrosine-kinase activity[6], which also has autophosphorylation activity[6,7]. It is interesting that, besides the epidermal growth factor receptor and insulin receptor, several oncogene products are also tyrosine kinases[8]. To reveal pos-

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sible homologies of the PDGF receptor with these and other proteins, to assist in the cloning of the gene(s) for this receptor and to understand its genetic regulation and mode of action, we wanted to isolate the receptor in sufficient quantities to obtain extensive amino acid sequence information.

Cells of the mouse fibroblast line NR6, which lack the structurally similar receptor for epidermal growth factor[9], were used as a source for the PDGF receptor. We did not have sufficient PDGF available nor did we have any anti-PDGF receptor antibodies to allow us to use affinity chromatography to isolate the receptor. However, successful isolation of hydrophobic membrane proteins and fragments thereof by size exclusion (SE)[10–14] and reversed-phase high-performance liquid chromatography (RP-HPLC)[15–18] has been reported. This paper describes a simple two-step liquid chromatographic method in which Triton X-100 and sodium dodecyl sulfate (SDS) detergents are used for microscale purification of a protein fraction with PDGF-dependent protein kinase activity. It is assumed that the protein showing the PDGF-dependent kinase activity is the PDGF membrane receptor protein, but no formal proof of this exists. For reasons of brevity, the protein fraction which we worked with will be designated as the "PDGF receptor". We also describe methods for the fragmentation of this highly insoluble protein, for the resolution of the resulting complex mixture of peptides by two-dimensional RP-HPLC and for the fractionation of large, denatured polypeptide fragments, all in preparation for automated sequencing. This methodology is generally applicable to large, hydrophobic proteins.

EXPERIMENTAL

Reversed-phase HPLC

The HPLC system used was identical to the system described by Tempst *et al.*[19] but was equipped with a Rheodyne Model 7125 sample injector with a 1-ml loop obtained from Alltech (Los Altos, CA, U.S.A.). All columns were of the analytical type (25 × 0.46 cm) and obtained from The Separations Group (Hesperia, CA, U.S.A.): C_4, Vydac 214 TP 54 with a 2 × 0.2 cm Upchurch C 130 B precolumn, handfilled dry with Vydac 214 TP B5 packing; C_{18}, Vydac 218 TP 54; diphenyl, Vydac 219 TP 54. Two different solvent systems were used, buffer A in each system being 0.1% trifluoroacetic acid (TFA) in water. In system I (for small to medium-sized peptides) buffer B consisted of 0.1% TFA in acetonitrile–water (70:30). Buffer B in system II (for larger polypeptides) was 0.1% TFA in acetonitrile–2-propanol–water (60:20:20). All experiments were carried out at ambient temperature, with flow-rates of 1 ml/min, unless stated otherwise, and with UV detection at 210 nm. Isolated fractions were collected manually.

High-performance size-exclusion chromatography (SE-HPLC)

The SE-HPLC system consisted of an Altex 110 A pump (Beckmann Instruments, Berkeley, CA, U.S.A.) with a 110-40 pressure filter, a Rheodyne Model 7125 sample injector with a 0.1-ml loop and an Hitachi Model 100-40 variable-wavelength detector (Cole Scientific, Calabasas, CA, U.S.A.) with a 12-μl flow cell. During protein purification, a TSK G4000SW column (30 × 0.75 cm), preceded by a Spherogel-TSK (7.5 × 0.75 cm) guard column, was equilibrated with 0.1% (v/v) SDS in 100 mM sodium phosphate, pH 7.0, at a flow-rate of 0.3 ml/min. Large polypeptide

fragments were fractionated on a TSK G2000SW column (60 × 0.75 cm), also with a Spherogel-TSK guard column, but were eluted with 6 M guanidine hydrochloride in 10 mM sodium phosphate (pH 7.0) at 0.2 ml/min. Experiments were conducted at ambient temperature with UV detection at 280 nm, and peak fractions were collected manually.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins and large polypeptide fragments were characterized or prepared for sequencing by SDS-gel electrophoresis on 0.5-mm or 0.7-mm thick polyacrylamide gels according to Laemmli[20]. Silver staining was performed as described by Merril et al.[21]. Quantification of the stained polypeptide bands was achieved with the aid of an automatic gel scanner/densitometer with integrator (Model 2202; LKB, Bromma, Sweden). Electroelution of proteins localized by either Coomassie staining or by autoradiography was performed essentially as described by Hunkapiller et al.[22]. Electroblotting of polypeptides onto activated glass-fiber sheets was carried out according to the method of Aebersold et al.[23].

Radioiodination

Proteins (3 μg), dissolved in 10 μl of 0.1% SDS, 100 mM sodium phosphate (pH 7.0), were reacted with 5 μl of Na[125]I (100 mCi/ml) and 5 μl of chloramine T at a concentration of 2 mg/ml. The reaction was terminated after 5 min by addition of 5 μl of sodium metabisulfite at a concentration of 2 mg/ml. Protein was separated from excess of reagents by SDS-PAGE. Autoradiography was performed by exposure of the gel or glass-fiber sheet to Kodak XAR-5 X-ray film at room temperature, or at −70°C, respectively. Activities of radiolabeled proteins in solution or in gel pieces were determined by gamma scintillation counting.

Reduction and alkylation of proteins

SDS-containing protein samples (1–2 ml) were made 8 M in urea, 10 mM in dithiothreitol (DTT) and 1 mM in EDTA, then dialyzed against 1 l of 350 mM Tris–HCl (pH 8.5), containing 8 M urea, 5 mM DTT, and 1 mM EDTA, for 16 h at room temperature under argon. The sample was then incubated for 30 min at 37°C under argon after additional, fresh DTT was added to a final concentration of 10 mM. Alkylation was performed by addition of recrystallized iodoacetamide (in 1 M sodium hydroxide) to a final concentration of 40 mM and reaction for 30 min at room temperature under argon in the dark. The reaction was stopped by raising the DTT concentration to 110 mM.

Protein cleavages

Tryptic digestion was performed in 100 mM ammonium bicarbonate, containing 1.8 M urea, for 5 h at 37°C. The protein concentration was approximately 45 μg/ml, and the enzyme/substrate ratio was 1:5 (w/w). An enzyme blank was incubated in parallel. TPCK-treated trypsin was obtained from Sigma (St. Louis, MO, U.S.A.).

Cyanogen bromide cleavage of 45 μg of protein was performed in 1 ml of 70% formic acid (Aldrich, Milwaukee, WI, U.S.A.) containing 0.7 M urea and 1 mg of cyanogen bromide (Baker, Phillipsburg, NJ, U.S.A.), for 24 h at room temperature under argon in the dark.

Amino acid analysis and sequencing

Total hydrolysis of protein was carried out by exposure of lyophilized protein to the vapor of 6 *M* hydrochloric acid for 24 h at 110°C in a sealed, evacuated glass vial (Millipore, Milford, MA, U.S.A.). Analysis was performed by the dabsyl chloride method[24].

Amino acid sequencing was performed on a Caltech automated gas-phase sequenator[25], the phenylthiohydantoin (PTH) amino acid identification being performed as described by Hunkapiller and Hood[26].

RESULTS

Protein fractionation

Mouse NR6 fibroblasts were cultured in roller bottles, and the membranes were solubilized using Triton X-100. Protein fractions enriched in PDGF receptor were then obtained by medium-performance affinity chromatography on a 30 × 1.6 cm column, containing a ricin toxin binding subunit attached to TSK-reactigel HW-65F (Pierce, Rockford, IL, U.S.A.), as described by Woo *et al.*[27].

Further purification of the PDGF receptor was first attempted by preparative SDS-PAGE followed by electroelution. The yield of PDGF receptor, calculated from the recovery of ^{32}P-phosphorylated protein, was only 5–10%. In addition, excessive amounts of SDS and Coomassie blue were present in the sample. A common procedure for the preparation of microgram amounts of proteins from such samples for the gas-phase sequenator is ion-pair extraction of the SDS into an organic phase while precipitating the protein[28]. However, we were not able to redissolve analytical quantities of this precipitated protein in aqueous solutions containing urea, guanidine hydrochloride, guanidine thiocyanate, SDS, Triton X-100 or in organic acids or bases. Therefore, an extensively dialyzed sample, containing approximately 50 pmoles of protein (by amino acid analysis), was analyzed in the gas-phase sequenator but yielded no sequence.

We then explored methodologies that did not involve drying or precipitation. Best results were obtained by the following procedure. Affinity column fractions containing PDGF-receptor activity were concentrated two-fold by using Centricon 30 (Amicon, Danvers, MA, U.S.A.) concentrators, made 2% (v/v) in SDS, then heated for 15 min at 37°C. Aliquots of 100 μl were chromatographed on a TSK G4000SW column with a mobile phase containing 0.1% SDS. Peak fractions were characterized by analytical SDS-PAGE, followed by silver staining. The results of SE-HPLC and SDS-PAGE are shown in Fig. 1. The off-scale peak at 33 min is due to Triton X-100 absorption at 280 nm. Fraction 2, indicated by the arrow, contained predominantly a MW 185 000 protein. It was concentrated (Centricon 30 at 20°C) and rechromatographed, using the same system and conditions. The chromatogram and SDS-PAGE analysis of the major peak (F) are presented in the central and right portions of Fig. 1, respectively. Besides a major band of MW 185 000, two minor bands were visible on the silver-stained gel. Densitometric scanning of the gel and integration of the peaks indicated that each of the contaminating bands had approximately 4% of the intensity of the major band. An aliquot of this final preparation was ^{125}I-labeled and analyzed on an SDS-gel under reducing conditions. Autoradiography revealed the same pattern and amounts of contamination, with an additional minor (<2%) band at MW 110 000 (data not shown).
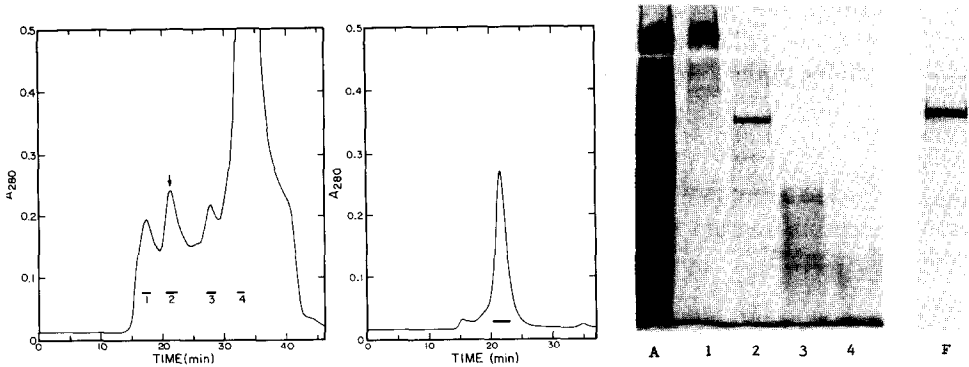
Fig. 1. Size-exclusion chromatography of lectin affinity-purified protein on a 30 × 0.75 cm TSK G4000SW column with Spherogel-TSK guard column, eluted with 0.1% SDS in 0.1 M sodium phosphate (pH 7.0) at 0.3 ml/min (left). Collected fractions 1–4 are indicated with heavy bars; 1% was electrophoresed on a 7.5% SDS polyacrylamide gel (right). Fraction 2 was concentrated and rechromatographed under the same conditions (middle) and analyzed by SDS-PAGE (far right, lane F). Lane A contains the initial affinity-purified (pre-SE-HPLC) material.

At this stage, we estimated, from the relative intensity of the Coomassie-stained bands on an SDS-gel compared to standards, that the total yield of SDS-purified MW 185 000 protein was 150 μg (800 pmoles), starting from 600 roller bottles of cultured mouse NR6 fibroblasts. Amino acid analysis confirmed this estimate, assuming that the glycoprotein contained approximately 35% carbohydrate by weight, consistent with the levels of glycosylation of other membrane glycoprotein receptors[10,29].

Study of the primary structure

A 300-pmole amount of the protein was reduced and carboxyamidomethylated in 350 mM Tris–HCl (pH 8.5), containing 2% SDS, and extensively dialyzed against 0.1% SDS. An attempt to obtain the amino-terminal sequence from this sample also yielded no data. We do not know whether this was due to blockage of the nascent protein in tissue culture or to artefactual blocking in the process of isolation.

In order to perform fragmentation of the protein and HPLC purification of the resulting peptides, specific precautions had to be taken to maintain the protein/peptides in solution and to minimize sample losses. Precipitation and drying steps were avoided, and the solubilization conditions were made compatible with fragmentation reactions and with HPLC of the resulting fragments. The results of a number of tests in this regard, with different denaturing agents, detergents, acids and bases, will be published elsewhere[30].

A 250-pmol amount of protein was reduced and alkylated by the method of Vehar *et al.*[31], but with modifications as described in the Experimental section. After the reaction was stopped, the mixture was dialyzed twice against 1 l of 4 M urea at room temperature for a total time of 12 h and subsequently twice against 2 M urea for the same period of time. The dialysis membrane had a molecular weight cut-off of 50 000, and mixed-bed ion-exchange beads (Bio-Rad AG 501-X8D, 20–50 mesh)

were added to the dialysis buffer to bind free cyanate. Dialysis of a radiolabeled analytical amount of the protein against 1 *M* urea resulted in complete precipitation. A 2-ml volume of the 2 *M* urea dialysate was concentrated to 0.6 ml in the Centricon 30 microconcentrator, and then 1 *M* ammonium bicarbonate was added to a final concentration of 100 m*M*. Trypsin digestion was then performed, as described in the Experimental section. After digestion the reaction mixture was immediately injected into a Vydac C_4 column, operated with solvent system I (see Experimental). The resulting chromatogram is shown in Fig. 1. One hundred and ten fractions were collected manually.
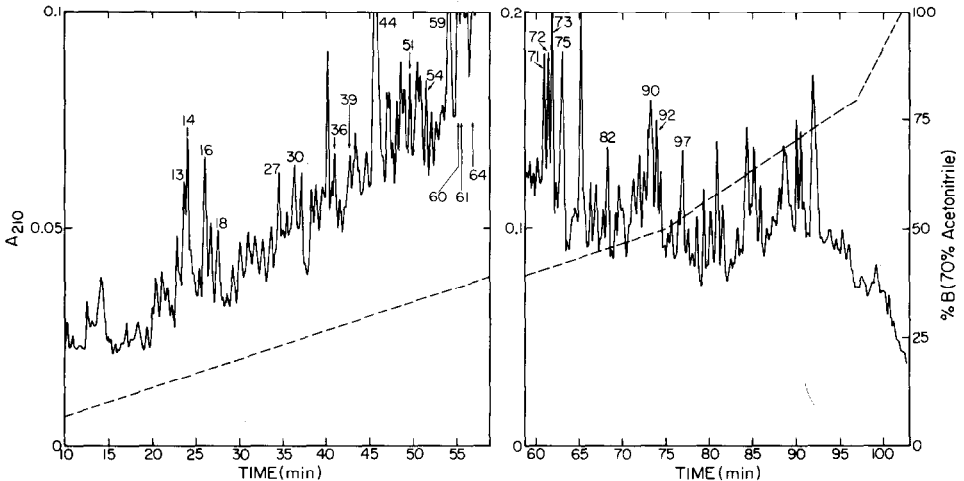

Fig. 2. Reversed-phase HPLC of a tryptic digest from 250 pmoles of protein on a Vydac C_4 column, with precolumn. Buffer system I (see Experimental section) was used at a flow-rate of 0.65 ml/min. The gradient was 0–50% B in 75 min, then 50–80% B in 22 min. Fractions were collected manually. Those indicated with a number were successfully sequenced after rechromatography.

Based on their highly symmetric peak shape, fractions 35 and 78 were selected for sequencing without further purification. However, both fractions turned out to be mixtures and no unambiguous sequences could be inferred from the data. Therefore, all remaining fractions were diluted two-fold with 0.1% TFA and rechromatographed on either a Vydac C_{18} column (fractions 12–41), a Vydac diphenyl column (fractions 59–105) or a Vydac C_4 column (fractions 42–58), using a shallower gradient in the case of the latter column as compared to the initial RP-HPLC experiment. Solvent system I was used in all experiments. Almost all of the original fractions were rechromatographed in this way and yielded two or more peptide peaks each. Selected results of these purifications are shown Fig. 3 (panels 1–7). All peptides recovered were homogeneous, as judged from subsequent sequencing data. An unambiguous sequence, ranging from 5 to 21 amino acids in length, was obtained for 23 peptides (numbered peaks in Fig. 2). Thus, a simple, two-dimensional RP-HPLC system, based on different Vydac columns but the same buffers, proved to be very effective
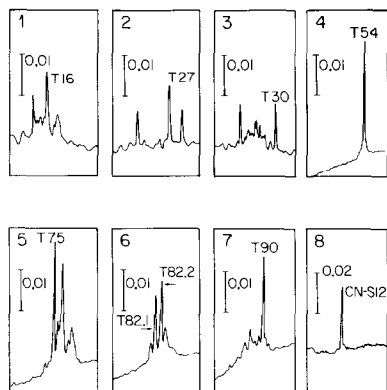
Fig. 3. Final purification of eight representative fractions from Figs. 2 and 4 on reversed-phase columns. Fractions T (tryptic) 16, 27, 30, 54, 75, 82 and 90 from the RP-HPLC experiment shown in Fig. 2 were diluted in an equal volume of 0.1% TFA and rechromatographed on either a Vydac C_{18} (1–3), diphenyl (5–7) or C_4 column (4) with mobile phase system I (see Experimental section). The gradients were 0–80% B in 80 min (1–3), 0–60% B in 120 min (4) and 15–80% B in 65 min (5–7). Peak CN-S12 (CNBr peptide, fraction 12 from the SE-HPLC experiment shown in Fig. 4) was injected into a Vydac C_4 column, operated with solvent system II (see Experimental section). The gradient used was 20–80% B in 60 min. UV detection, in all cases, was at 210 nm.

for the nearly complete separation of the complex tryptic peptide mixture of this MW 185 000 membrane protein.

Cyanogen bromide cleavage was performed on 250 pmoles of receptor protein. Since we expected the cleavage to yield a number of large fragments that would be analyzed and/or isolated by SDS-PAGE, we included an analytical amount of radiolabeled protein in the sample to allow us to perform autoradiography. To this end, 3 μg of TSK G4000SW-purified material was iodinated and the reaction mixture was loaded on a 7.5% SDS gel (0.5 mm thick). Bands were identified by autoradiography, and the MW 185 000 major protein was cut out and eluted by shaking it for 2 h at 37°C in 0.5 ml of 100 mM sodium phosphate buffer, containing 2% SDS. We recovered approximately 65% of the iodinated material, and all counts were trichloroacetic acid (TCA)-precipitable. An aliquot from this solution, containing 10^6 cpm, was mixed with the 250-pmole sample, which was then reduced, carboxyamidomethylated and dialyzed as described for the tryptic digest. Concentrated (96%) formic acid was then added to the sample, which was dissolved in 0.3 ml of 2 M urea, to yield a final acid concentration of 70%; then 1 mg of CNBr was added. After digestion for 24 h at 22°C, the sample was frozen on solid carbon dioxide and concentrated to 50 μl in a rotary evaporator (Savant, Hicksville, NY, U.S.A.). Water (150 μl) was added and the mixture was stirred and heated at 37°C until the solution was clear. The sample was then chromatographed on a TSK G2000SW column, equilibrated in 10 mM sodium phosphate (pH 7.0) containing 6 M guanidine hydrochloride. These chromatographic conditions allow optimum separation of polypeptides in the 1000–10 000 molecular-weight range[30,32]. Radioactivity was eluted with all nine major peaks (indicated in Fig. 4), and the recovery was nearly complete. No radiolabeled material was monitored beyond fraction 14.
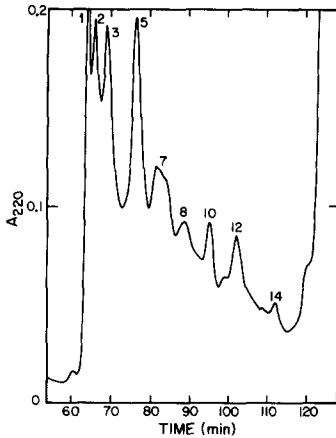
Fig. 4. Size-exclusion chromatography of a cyanogen bromide digest from 250 pmoles of protein on a 60 × 0.75 cm TSK G2000SW column with Spherogel-TSK guard column, eluted with 6 M guanidine hydrochloride in 10 mM sodium phosphate (pH 7.0) at 0.2 ml/min.

Fractions 5–14 were rechromatographed without any further treatment on a Vydac C_4 column, employing solvent system II. It has previously been shown that acetonitrile containing 2-propanol, as an organic modifier, gives good recovery and resolution in RP-HPLC of large, denatured polypeptides[17,18]. Fractions 10 and 12, on rechromatography, gave excellent peak shape and high (>80%) recovery. The result of the latter experiment can been seen in Fig. 3, panel 8. Recovery of peaks 5, 7, 8 and 14 from the C_4 column was low (<5%), probably due to irreversible binding to the stationary phase. The column was treated with acetic acid–guanidine hydrochloride[30], but no further protein could be recovered. Fractions 1–3, expected to be larger than MW 10 000, were dialyzed against 4 M urea, then 2 M urea and finally 0.1% SDS, each for a period of 12 h at room temperature. Each sample, of approximately 1.5 ml, was then concentrated to 100 μl in the Centricon 10 microconcentrator and electrophoresed in a 10% SDS gel (0.7 mm thick). The SDS gel was subsequently subjected to electroblotting at pH 8.3, according to the method of Aebersold *et al.*[23]. Autoradiography of the blot revealed three distinct bands of apparent molecular weights 185 000 (uncleaved protein), 90 000 and 60 000, respectively.

DISCUSSION

We have developed, a rapid, simple two-step purification procedure, employing affinity chromatography on a ricin B column in the presence of Triton X-100, followed by SE-HPLC in the presence of SDS, to yield a MW 185 000 polypeptide that was *ca.* 92% pure. Since we were unable to obtain an amino-terminal sequence from the intact protein, internal fragments had to be prepared and isolated for automated sequencing. The combination of methods we have developed is generally applicable to subnanomole amounts of large, hydrophobic membrane proteins with blocked amino-termini. By adapting existing methods of protein fragmentation to our sparingly soluble receptor polypeptide and by using a two-stage RP-HPLC approach, we

have been able to produce and resolve more than 100 tryptic peptides, 23 of which have yielded an unambiguous protein sequence. This method represents a very useful addition to existing procedures for the resolution of peptides[17,33,34] and improves the versatility of RP-HPLC for the fractionation of very complex peptide mixtures present in low amounts (100–200 pmoles). Excellent results in the micropreparative isolation of large CNBr fragments for sequencing purposes were obtained by another two-dimensional separation method. The complete mixture was first size-fractionated by SE-HPLC under strong denaturing conditions (6 M guanidine hydrochloride), and then the peptides smaller than MW 10 000 were immediately subjected to RP-HPLC, using acetonitrile–2-propanol gradients. Alternatively, larger peptides (MW > 10 000) were subjected to SDS-PAGE in a second purification, followed by electroblotting onto activated glass-fiber sheets[23]. Both approaches yielded polypeptides that could be used for automated sequencing without any additional treatment. Twelve CNBr peptides were fractionated and purified to homogeneity by this method.

Starting with a total amount of 500 pmoles of the putative PDGF receptor for a tryptic digest and CNBr cleavage and using the methodology described above, we were able to sequence a total number of 232 amino acids; the stretches of sequence range from 5 to 21 residues.

Using the protein sequence data obtained, we have designed and synthesized oligodeoxynucleotide probes to be used in the cloning of the gene coding for the PDGF receptor. We expect that the combination of protein chemical and molecular genetic approaches we have developed will result in a substantial improvement in our understanding of the PDGF receptor and of other hydrophobic membrane proteins.

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