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Protamine Inhibits Platelet Derived Growth Factor Receptor Activity but not Epidermal Growth Factor Activity

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Protamine sulfate blocked ^{125}I-PDGF binding to its specific physiological receptor on Swiss mouse 3T3 cells. Reduced ^{125}I-PDGF binding in the presence of protamine sulfate correlated directly with a protamine sulfate dose-dependent decrease in the PDGF-dependent incorporation of [^{3}H]-thymidine into 3T3 cells and a decreased PDGF-stimulated tyrosine-specific protein kinase activity in isolated membrane preparations of 3T3 cells. Protamine sulfate blocked ^{125}I-PDGF binding to simian sarcoma virus transformed cells (SSV-NIH 3T3 and SSV-NP1 cells) and to nontransformed cells in a manner qualitatively identical to unlabelled PDGF. In contrast, protamine sulfate enhanced the specific binding of ^{125}I-EGF by increasing the apparent number of EGF receptors on the cell surface. The increase in ^{125}I-EGF receptor binding was not prevented by cycloheximide nor by actinomycin D. Protamine sulfate did not affect ^{125}I-EGF binding to membranes from 3T3 cells or the EGF-stimulated 3T3 cell membrane tyrosine specific protein kinase activity, suggesting that protamine sulfate may have exposed a population of cryptic EGF receptors otherwise not accessible. Protamine sulfate was fractionated into four active fractions by Sephadex G-50 gel filtration columns; the half maximum inhibition concentration of ^{125}I-PDGF binding to 3T3 cells of protamines I and II (MW ~ 11,000 daltons and 7,000 daltons, respectively) is ~ 0.4 μM. Protamine II (MW ~ 4,800 daltons) was equally active (half maximum inhibition concentration ~ 0.4 μM); protamine IV (MW ~ 3,300 daltons) was substantially less active (half maximum inhibition concentration ~ 2.8 μM).

These investigations have extended previous observations that protamine sulfate is a potent inhibitor of PDGF binding and establish that protamine sulfate blocks PDGF binding at the physiological receptor, preventing PDGF initiated biological activities. Protamine sulfate can be used as a reagent to separate the influence of PDGF and EGF on cells with high specificity and has been used to demonstrate that the receptors on simian sarcoma virus transformed 3T3 cells qualitatively respond identically to protamine sulfate as to unlabelled PDGF and are likely identical to those on nontransformed 3T3 cells.

Key words: PDGF, EGF, receptor, oncogenes

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The platelet derived growth factor (PDGF) is the principal mitogenic protein in human serum for Swiss mouse 3T3 cells [1]. PDGF also is a potent chemoattractant protein for monocytes, neutrophils, fibroblasts, and smooth muscle cells [2–5]. Because PDGF is released when platelets are activated, PDGF is considered likely to be an important mediator of inflammatory and repair responses at sites of injured blood vessels and may be important also in the pathological processes of atherosclerosis in humans [6,7].

A partial amino acid sequence of PDGF was shown recently to be highly homologous with that predicted for $p28^{v\text{-}sis}$, the putative transforming protein of the simian sarcoma virus (SSV) [8,9]. A growth factor identical to PDGF in assays of mitogenic activity and antigenically indistinguishable from PDGF subsequently was identified in SSV-transformed but not in control 3T3 cells [10], suggesting that PDGF or a closely related protein may be required for transformation by SSV. This protein $p28^{v\text{-}sis}$ or its processed product, has been identified also in conditioned media from cultures of SSV-transformed cells and appears to interact with SSV-transformed cell receptors to stimulate [^{3}H]-thymidine uptake, suggesting that the transforming protein of SSV may serve as an autocrine regulator of cell growth [J.S. Huang, S.S. Huang, and T.F. Deuel, Cell: 39:79–87, 1984]. It appears that $p28^{v\text{-}sis}$ is processed in transformed cells by glycosylation and proteolytic degradation [11]. The predominant form of $p28^{sis}$ identified by immunoprecipitation with anti-PDGF antisera in SSV-transformed NIH 3T3 cells was ~ 20 kd [10].

Specific PDGF receptors have been identified on 3T3 cells, human skin fibroblasts, arterial smooth muscle cells, as well as on a variety of other cells derived from connective tissues [12–15]. It seems very likely that PDGF and $p28^{v\text{-}sis}$ bind to the same receptors. A partially purified extract of $p28^{sis}$ has been shown to compete directly with ^{125}I-PDGF for receptor binding [J.S. Huang, S.S. Huang, and T.F. Deuel, unpublished data]. The specific interactions of PDGF and $p28^{v\text{-}sis}$ with cell surface receptors has become a major focus of research; understanding these interactions should provide the initial basis for understanding the mechanisms of action of each protein. The authors recently reported the purification of the PDGF-receptor protein from Swiss mouse 3T3 cells and were able to identify protein tyrosine kinase activity specifically associated with the purified receptor protein [16]. During the course of these experiments, protamine sulfate was used as a specific inhibitor of ^{125}I-PDGF binding to its receptor. Protamine sulfate also has been used to specifically block PDGF stimulated chemotaxis [2] and to block the stimulation by $p28^{sis}$ in conditioned media of SSV-transformed cells of [^{3}H]-thymidine incorporation into 3T3 cell DNA. Thus, protamine has been used as an important reagent for modulating PDGF receptor activity, as suggested by the original observation that it is a potent competitive inhibitor of ^{125}I-PDGF binding to 3T3 cells [14].

It has not been established that protamine competes with ^{125}I-PDGF for binding to its physiological receptor and that protamines are able to block physiological responses in target cells, nor have the active fractions of heterogeneous protamine sulfate been identified. The present experiments were conducted to extend previous observations on the effect of protamine sulfate in modulating PDGF activity on cell surfaces and now show that the effect of protamine sulfate is to block specifically the binding of PDGF to its physiological cell surface receptor and to prevent receptor-mediated biological activities in 3T3 cells. In addition, protamine sulfate also now has been shown to have a remarkable differential effect on EGF (epidermal growth

factor) and PDGF binding; under conditions in which PDGF binding to cells is sharply reduced, protamine sulfate enhances somewhat the binding of EGF to its receptor. The active fractions of protamine sulfate have been separated and partially characterized. The utility of protamine sulfate has been extended to show that it qualitatively is identical to unlabelled PDGF in blocking ^{125}I-PDGF binding to SSV-transformed cells as well. The results thus provide the means for modulating PDGF activity on cells and complement results of other laboratories showing that protamine sulfate is a specific inhibitor of tumor angiogenesis [17], an agonist in stimulating limited DNA synthesis in cells [18], and an agonist in stimulating protein kinase activity [19].

MATERIALS AND METHODS

Swiss mouse 3T3 fibroblasts (CLL 92) were obtained from the American Type culture collection. Human epidermal carcinoma A431 cells were obtained through the courtesy of Dr. Luis Glaser (Department of Biological Chemistry, Washington University School of Medicine, St. Louis, MO). Simian sarcoma virus (SSV) transformed NIH 3T3 cells were kindly provided by Dr. S.A. Aaronson. SSV transformed NP1 cells, (marmoset fibroblasts) were the gift of Dr. F. Wong-Staal, NIH. Cell culture cluster plates (24 wells, 16 mm well diameter) were obtained from Costar. Na^{125}I (17 Ci/mg) and [γ-^{32}P]-ATP (5-10 Ci/mmol) were purchased from New England Nuclear. Protamine sulfate (salmon, lot 266-9520), cycloheximide (lot 32C-284), and DNA-cellulose (lot 70F-8100) were obtained from Sigma. Mouse epidermal growth factor was obtained from Collaborative Research. Human platelet derived growth factors I and II were prepared as previously described [20]. Each protein was found to be pure in SDS (sodium dodecyl sulfate) gel electrophoresis and to have equal mitogenic potency [20]. Protein markers (phosphorylase b, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme) were obtained from Bio-Rad. Actinomycin D was obtained from P-L Biochemicals, Inc.

Cell Culture and Membrane Preparation

Swiss mouse 3T3 fibroblasts, SSV-NIH 3T3 cells, SSV-NP1 cells, and human epidermal carcinoma A431 cells were grown to confluence in Costar 24-well cluster dishes in DME medium (Dulbecco's modified Eagle's medium) containing 10% fetal calf serum. A plasma membrane enriched fraction from Swiss mouse 3T3 fibroblasts was prepared as previously described [21]. Membranes were stored at −70°C.

Iodination of PDGF

PDGF II was iodinated by the Iodogen method, as previously described [14]. ^{125}I-PDGF (~ 16 uCi/μg) was stored at −20°C in 0.1 M acetic acid containing 1 mg/ml of human serum albumin.

Iodination of EGF

EGF was iodinated with slight modifications by the chloramine-T method according to the procedure of Comens et al [22]. Four μg EGF was mixed with a solution containing 100 μl of 0.3 M sodium phosphate buffer (pH 7.5) and 20 μl of Na^{125}I (1.5 mCi, carrier free). Two hundred μg of chloramine T was then added. After 40 sec incubation at room temperature, 20 μl of metabisulfite solution (10 mg/ml sodium metabisulfite, 38 mg/ml sodium iodide) was added to stop the reaction.

About 0.35 ml of 0.1% bovine serum albumin in 0.2 M sodium phosphate buffer (pH 7.4) was mixed immediately with the above reaction mixture, and the solution was applied on a Sephadex G-25 column (0.9 × 49 cm) equilibrated with 0.1% bovine serum albumin in 0.2 M sodium phosphate buffer (pH 7.4) to remove free iodide. ^{125}I-EGF (150 μCi/μg) was stored at −20°C before use.

Binding of ^{125}I-PDGF to 3T3 Fibroblasts

The binding experiments with ^{125}I-PDGF were essentially as previously described [14]. The volume of assay medium was 0.5 ml instead of 1 ml, in 5 mM HEPES (N-(2-hydroxyethyl)-1-piperazine-N-2 ethansulfonic acid) (pH 7.4) containing 5% plasma derived serum and 0.15 M NaCl.

Binding of ^{125}I-EGF to 3T3 Fibroblasts and Human A431 Cells

Swiss mouse 3T3 fibroblasts and human A431 cells were grown to confluence in Costar 24-well cluster dishes (16 mm well diameter) in DME medium containing 10% fetal calf serum. Confluent monolayer cultures were washed twice with 1 ml of PBS (phosphate buffer saline). Half ml of binding medium was added to each well. The binding medium consisted of ^{125}I-PDGF, protamine sulfate, and 0.1% human serum albumin in 5 mM HEPES buffer, 0.15 M NaCl (pH 7.4). Binding experiments were terminated after 1 hr by washing three times with PBS. Equilibrium of binding was established at 1 hr by multiple time course analyses. The cells in each well were solubilized with 0.5 ml of 0.4 M NaOH. One quarter ml from each well was taken for measurement of radioactivity. Specific binding was the difference between binding in the presence and absence of 4 μg unlabelled EGF.

Membrane Phosphorylation

Phosphorylation experiments with [γ-^{32}P]-ATP were carried out as described previously [21]. The reaction mixture (50 μl) consisted of 40 μg membrane, 300 ng/ml PDGF or various concentrations of EGF, 10 μg bovine serum albumin (protein carrier), and protamine sulfate as indicated in 20 mM HEPES buffer containing 20 mM $MgCl_2$. The reaction mixture was preincubated for 30 min at 0°C. Twenty μM of [γ-^{32}P]-ATP was then added. After 10 min at 4°C, the reaction was terminated with 10% TCA. The TCA precipitate then was subjected to SDS gel electrophoresis. Stained gels were dried and subjected to autoradiography for 1–3 days at −70°C with intensifier screens, using Kodak X-Omat AR film. Bands with ^{32}P-labelled proteins (M_r ~ 170,000) were excised from dried gels, solubilized in 30% H_2O_2 at 80°C, and assayed for radioactivity.

Purification of Protamine Sulfate From Salmon Sperm

Fifty mg of protamine sulfate was dissolved in formic acid/acetic acid/water (25:87:888, by volume) and subjected to gel filtration with Sephadex G-50 (superfine 1.5 × 90 cm) equilibrated with the same solvent. Protamine I molecular weight (MW ~ 11,000), protamine II (MW ~ 7,200), protamine III (MW ~ 4,800), and protamine IV (MW ~ 3,300) were obtained by rechromatography of protamine fractions obtained from the first column chromatography on the same Sephadex G-50 column.

Protein Determination

Protein concentration was determined by the Bio-Rad protein microassay [23] or by the method of Lowry et al [24], using bovine serum albumin as standard.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out according to Laemmli [25], using 7.5% gels for ^{32}P-autoradiograms and 15% gels for estimation of homogeneity of purified protamine sulfate fractions. Small polypeptides (Boehringer Mannheim) were used as standard MW markers: insulin B chain (MW 3,400), aprotinin (MW 6,500), cytochrome C (MW 12,500), and soybean trypsin inhibitor (MW 21,500).

Mitogenic Assay

The mitogenic assay measured the PDGF-dependent incorporation of [^{3}H]-thymidine into TCA precipitable material in quiescent Swiss mouse 3T3 fibroblasts [26]. Monolayer cell cultures were incubated with 20 ng/ml of PDGF and with various concentrations of protamine sulfate in DME medium, containing 5% plasma derived serum. After incubation at 37°C for 20 hr, the assay was stopped by washing with PBS twice. One ml of cold TCA was then added. After 10 min, the TCA precipitable material was washed with 1 ml of ethanol:ether (2:1) and dissolved in 0.4 ml of 0.4 M NaOH to measure radioactivity.

RESULTS

Previously, it was shown that protamine sulfate was a competitive inhibitor of ^{125}I-PDGF binding to Swiss mouse 3T3 cells. This investigation next tested protamine sulfate as an inhibitor of ^{125}I-PDGF binding to cells transformed by simian sarcoma virus (SSV). The SSV transforming protein ($p28^{v\text{-}sis}$) has striking amino acid sequence homology with PDGF [8,9], and cells transformed by SSV appear to have markedly reduced numbers of receptors for ^{125}I-PDGF. Whether these receptors are similar to receptors on nontransformed cells has not been established. Competition between ^{125}I-PDGF and protamine sulfate for binding to nontransformed 3T3 cell receptors was demonstrated previously [14]. The inhibition of ^{125}I-PDGF binding by protamine sulfate then was tested with SSV-transformed NIH 3T3 cells as one criteria to assess the properties of the two receptor populations (Fig. 1). Protamine sulfate was a potent inhibitor of ^{125}I-PDGF binding to SSV-transformed cells (Fig. 1). The dose response curve was essentially identical to that previously observed with nontransformed 3T3 cells [14] and in both instances was qualitatively identical to the competitive binding of ^{125}I-PDGF by unlabelled PDGF, suggesting that the limited number of PDGF receptors on transformed cells are similar to those on nontransformed cells. ^{125}I-PDGF and partially purified $p28^{v\text{-}sis}$ also compete for receptor binding on SSV-NIH 3T3 cells; the kinase activity of the receptor on SSV-NIH 3T3 cells is intact (data not shown), suggesting further that $p28^{v\text{-}sis}$ and PDGF compete for the same receptor and raising the possibility that $p28^{v\text{-}sis}$ secreted by transformed cells may feed back by an autocrine mechanism to stimulate the growth of SSV-transformed cells. A similar K_d for the binding of ^{125}I-PDGF to transformed and nontransformed cells has been reported also [27].

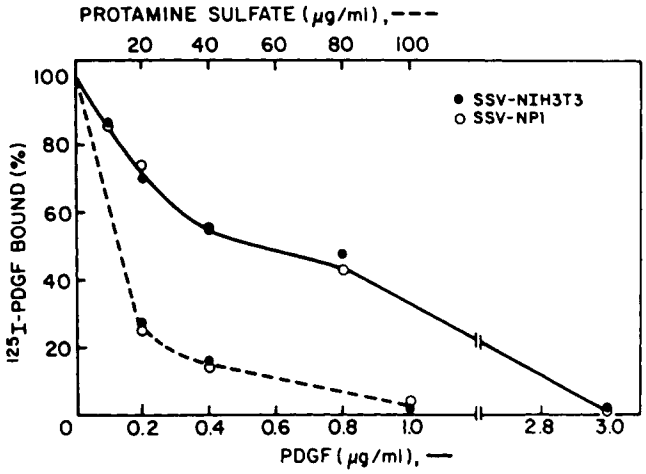


Fig. 1. Inhibition of ^{125}I-PDGF binding to SSV-NP1 and SSV-NIH 3T3 cells by unlabelled PDGF and protamine sulfate. The binding medium contained 100 ng/ml (3.3 nM) ^{125}I-PDGF and various concentrations of unlabelled PDGF or protamine sulfate. The specific binding of ^{125}I-PDGF to SSV-NP1 and SSV-NIH 3T3 cells were 3714 cpm/well and 3098 cpm/well, respectively, and were taken as 100% of binding.

Evidence was then sought to establish that protamine sulfate blocked binding of ^{125}I-PDGF to its physiological receptor, as opposed to a receptor-like binding site not mediating biological activities. PDGF is the principal mitogen in human serum for cells of mesenchymal origin; mitogenic stimulation serves as an indicator of PDGF binding to its physiological cell surface receptor. PDGF stimulation of [^{3}H]-thymidine incorporation into cells ± protamine sulfate was examined (Fig. 2). In our assay, the incorporation of [^{3}H]-thymidine into 3T3 cells is related linearly to PDGF concentrations from 0–20 ng/ml [26]. At 20 ng/ml PDGF, about 80,000 dpm of [^{3}H]-thymidine was incorporated into 3T3 cells; this incorporation was equivalent to 60–70% of the positive control (16% human serum) in repeated experiments. Increasing concentrations of protamine sulfate quantitatively reduced [^{3}H]-thymidine incorporation into 3T3 cells (Fig. 2). Fifteen percent inhibition of [^{3}H]-thymidine occurred at 20 μg/ml protamine sulfate; at 80 μg/ml, 45% inhibition was found. Protamine sulfate was not able itself to stimulate [^{3}H]-thymidine incorporation into 3T3 cell DNA. Higher concentrations of protamine sulfate (> 100 μg/ml) were tested and found to reduce further [^{3}H]-thymidine incorporation, but irregularly. This further reduction was complicated by apparent toxicity of protamine sulfate and was not tested in more detail. Previously, it had been shown that 60 μg/ml protamine sulfate resulted in more than 95% inhibition of ^{125}I-PDGF binding to 3T3 cells [14]. The quantitative differences in the effects of protamine sulfate on binding and on mitogenic activity likely reflect the substantially longer periods of incubation of PDGF in the mitogenic assay (1 hr vs 20 hr.)

PDGF stimulates the tyrosine specific phosphorylation of an ~ 170 kd protein in membranes from Swiss mouse 3T3 cells and from human fibroblasts [21,28]; protamine sulfate was tested to see if the PDGF-stimulated protein kinase activity was blocked in parallel with the inhibition of ^{125}I-PDGF binding. PDGF stimulated tyrosine specific incorporation of ^{32}P into the 170 kd protein in 3T3 cell membranes

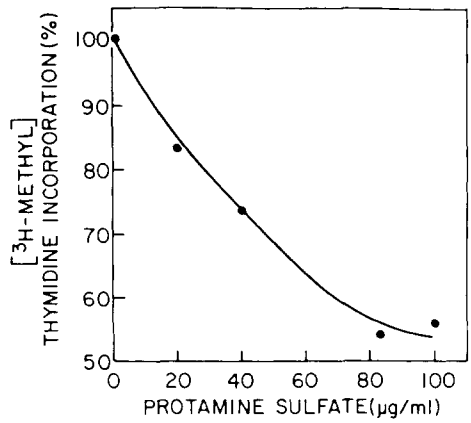


Fig. 2. Effect of protamine sulfate on PDGF stimulated [^{3}H]-thymidine incorporation of Swiss mouse 3T3 cells. Confluent monolayer cultures of 3T3 cells were incubated with 20 ng of PDGF, 0.2 uCi [^{3}H]-thymidine, and various concentrations of protamine sulfate in 1 ml of 5 percent plasma derived serum in DME medium. After incubation at 37°C for 20 hr, the monolayers were washed with PBS twice, precipitated by 10% TCA, and washed with ethanol/ether (2:1). The radioactivity was estimated by dissolving the TCA-precipitable substance in 0.4 M NaOH and counting with a scintillation counter. The radioactivity incorporated (83092 dpm) without protamine sulfate was taken as 100%.

was progressively blocked by increasing concentrations of protamine sulfate, as indicated by measuring ^{32}P incorporation into the 170 kd protein after excision from SDS gels (Fig. 3). Approximately 60% inhibition was found at 50 μg/ml protamine sulfate and 80% inhibition at 100 μg/ml. Repeat experiments gave similar results.

We next analyzed the binding of ^{125}I-EGF to cells to determine if the inhibition of ^{125}I-PDGF binding by protamine sulfate was specific for the PDGF receptor. Figure 4 demonstrates an increase of ~ 65% in ^{125}I-EGF (1 ng/ml) binding to 3T3 cells at protamine sulfate concentrations of 10 μg/ml and an 80% increase at 20 μg/ml. This unexpected result then was tested with a second cell type, the A431 cell. A431 cells have very high numbers of EGF receptors/cell [29]; the binding of ^{125}I-EGF to A431 cells was increased reproducibly by protamine sulfate, although the increase in binding was substantially less than with Swiss mouse 3T3 cells. Nearly a 25% increase in ^{125}I-EGF binding was demonstrated at 40 μg/ml (Fig. 5). Repeated experiments gave identical results.

The increase in ^{125}I-EGF binding to cells in the presence of protamine sulfate was measured at concentrations of ^{125}I-EGF well below saturation; the observed increase of ^{125}I-EGF binding could be explained by an increase in EGF receptor number or by a decrease in the K_d of receptor ^{125}I-EGF binding. Direct binding of ^{125}I-EGF ± protamine sulfate to Swiss mouse 3T3 cells was measured; Scatchard plots showed protamine sulfate increased ^{125}I-EGF receptor numbers, but the apparent K_d for ^{125}I-EGF binding was not modified significantly by protamine sulfate (Figs. 6a,b). The apparent increase in EGF-receptor number in the presence of protamine sulfate was not influenced by incubation with cycloheximide or with actinomycin D (Table I), suggesting the increase in EGF receptor number is not the result of new synthesis of EGF receptors. The increased number of receptors likely arises from exposure of otherwise cryptic receptors or from receptors previously not

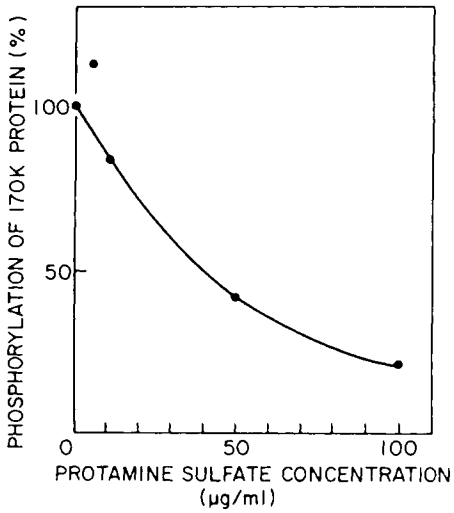


Fig. 3. Effect of protamine sulfate on PDGF stimulated phosphorylation of Swiss mouse 3T3 cell membranes. The phosphorylation assays were performed at various concentrations of protamine sulfate in the presence and absence of 300 ng/ml of PDGF as described in Methods. The radioactivity was visualized by autoradiography using Kodak X-Omat AR films. The bands of the 170 kd protein were excised and radioactivity was measured. The PDGF-stimulated phosphorylation (1090 cpm) of the 170 kd protein in the absence of protamine sulfate was taken as 100%.

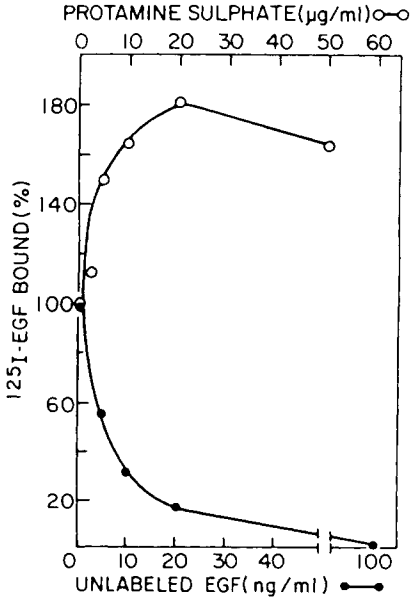


Fig. 4. Effect of protamine sulfate and unlabelled EGF on the binding of ^{125}I-EGF to Swiss mouse 3T3 fibroblasts. Monolayer cell cultures were incubated with 0.5 ml of assay medium containing 1 ng/ml of ^{125}I-EGF and various concentrations of protamine sulfate or unlabelled EGF as indicated in 5 mM HEPES buffer (pH 7.4), 0.15 M NaCl, and 0.1% human serum albumin. The specific binding (852 cpm/well) of ^{125}I-EGF in the absence of protamine sulfate was taken as 100% binding.

TABLE I. Effect of Cycloheximide and Actinomycin D on the Increase of ^{125}I-EGF Binding to 3T3 Cells Mediated by Protamine Sulfate*

Addition	Specific binding to 3T3 cells (cpm/well)	
	No Protamine sulfate	Protamine sulfate (100 μg/ml)
None	2028	3142
Cycloheximide (50 μg/ml)	2126	3308
Actinomycin D (100 μg/ml)	2186	3252

*A half ml of assay medium consisted of 2 ng of ^{125}I-EGF (1×10^5 cpm/ng), 50 μg/ml of cycloheximide or 100 μg/ml of actinomycin D in 5 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl and 0.1% human serum albumin. After incubation at 37°C for 1 hr, the binding assays were terminated by washing with 1 ml of PBS three times. The specific binding was measured from the difference in the absence and presence of 4 μg/ml of unlabelled EGF.

in a conformation favoring ^{125}I-EGF binding. Membrane fractions isolated from 3T3 cells [21] were tested and ^{125}I-PDGF binding measured under conditions used with intact cells except for suspension of membrane fragments and separation by centrifugation. No effect of protamine sulfate (20 μg/ml) on ^{125}I-EGF binding to isolated 3T3 cell membranes was found at 0.5 and 1 ng/ml ^{125}I-EGF (data not shown). Thus, the protamine sulfate effect is limited to the intact surface of 3T3 cells and is not observed on isolated membrane preparations.

EGF also stimulates ^{32}P-incorporation from [γ-^{32}P]-ATP into tyrosine residues of a membrane-bound protein in Swiss mouse 3T3 cells; this protein is the EGF receptor protein [30]. The effect of protamine sulfate on the EGF-dependent phosphorylation in membranes from Swiss mouse 3T3 cells was tested to establish that protamine generally did not inhibit protein tyrosine kinase activity. In striking contrast to results with PDGF, no effect of protamine sulfate could be demonstrated on EGF-stimulated ^{32}P incorporation (Fig. 7), providing additional evidence that protamine sulfate specifically affects ^{125}I-PDGF but not ^{125}I-EGF receptor-binding. Protamine sulfate in the absence of PDGF or EGF is without effect on endogenous membrane protein kinase activity (results not shown).

Protamine sulfate as commercially obtained is not pure; fractionation with Sephadex G-50 helped define fractions active in reducing ^{125}I-PDGF binding to 3T3 cells (Fig. 8a). Rechromatography of peaks resulted in a marked improvement of purity of individual fractions (Fig. 8b). SDS gel electrophoresis analysis (Fig. 9) of these separated fractions shows essential homogeneity of separated fractions protamine III and protamine IV (Fig. 9, columns D,E). The effectiveness of individual protamine fractions to inhibit ^{125}I-PDGF binding was measured (Fig. 10). Protamine I and II (MW ~ 11kd and 7.2kd, analyzed together) and protamine III (MW ~ 4.8kd) were very effective inhibitors. Fifty percent inhibition of ^{125}I-PDGF binding was observed at 0.4 μM. Protamine IV (MW 3,300kd) was far less effective; ~10 μg/ml (2.7 μM) was needed to achieve 50% inhibition of ^{125}I-PDGF binding to 3T3 cells. Other fractions were without activity.

Protamine III was tested separately for its effect on binding of ^{125}I-EGF and ^{125}I-PDGF to 3T3 cells and its effect on PDGF- and EGF-stimulated [^{3}H]-thymidine incorporation and protein phosphorylation under conditions described in Figures 1–7. Protamine III was found to produce identical results to those found above at concen-

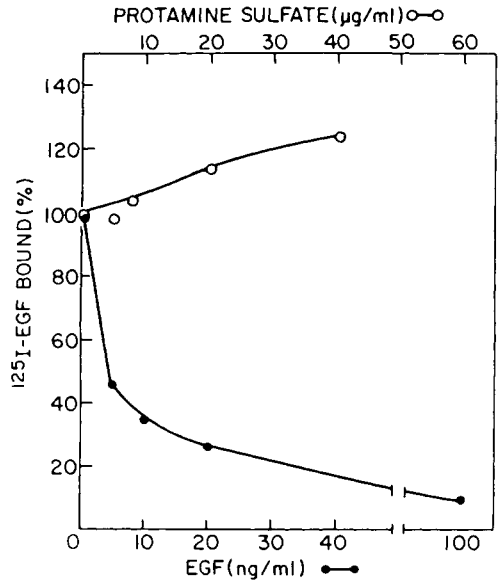
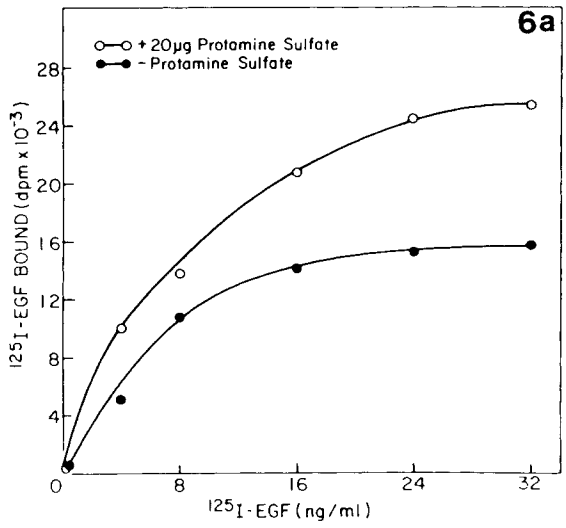


Fig. 5. Effect of protamine sulfate and unlabelled EGF on the binding of ^{125}I-EGF to human A431 cells. Monolayer cell culture was incubated with 0.5 ml of assay medium containing 0.8 ng/ml of ^{125}I-EGF and various concentrations of protamine sulfate or unlabelled EGF as indicated in 5 mM HEPES buffer (pH 7.4), 0.15 M NaCl and 0.1 % human serum albumin. The specific binding (80808 cpm/well) in the absence of protamine sulfate was taken as 100% binding.

trations ~ 20% of those required for similar effects using unpurified protamine mixtures. Thus, protamine III is a highly effective purified protein capable of competing directly with PDGF for the physiological PDGF receptor.

DISCUSSION

The binding studies and physiological responses of cells to PDGF and EGF as modified by protamine sulfate described here have extended substantially original observations that protamine sulfate blocks ^{125}I-PDGF binding to 3T3 cells by competitive inhibition. Protamine sulfate now has been shown to block ^{125}I-PDGF binding to the receptor on transformed cells with a dose response identical qualitatively to unlabelled PDGF and to that previously shown with nontransformed cells, suggesting that the receptors on transformed and on nontransformed cells are the same. The results also show that protamine sulfate blocks ^{125}I-PDGF binding to its physiological PDGF receptor on 3T3 cells. Protamine sulfate thus blocks the PDGF-dependent stimulation of tyrosine specific protein kinase activity and the PDGF stimulation of [^{3}H]-thymidine incorporation into DNA in parallel with decreased ^{125}I-PDGF binding of the 3T3 cell. Evidence for specificity of the effect of protamine sulfate on ^{125}I-PDGF binding also has been obtained in these studies. In contrast to results with ^{125}I-PDGF, a moderate increase in the binding of ^{125}I-EGF was found. This increase



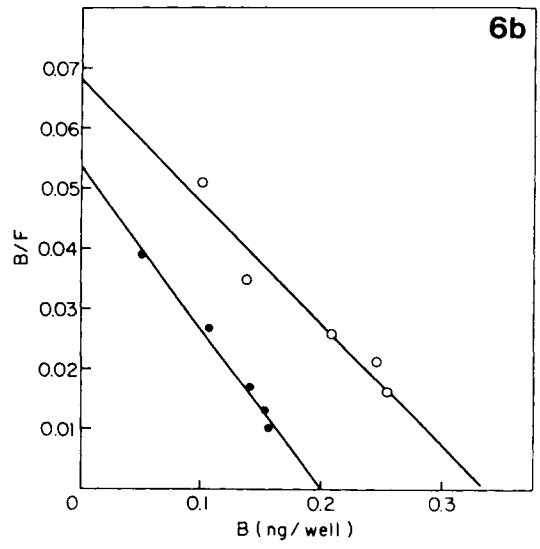


Fig. 6a. Concentration dependence of ^{125}I-EGF binding to Swiss mouse 3T3 cells in the presence and absence of protamine sulfate. Various concentrations of ^{125}I-EGF were incubated with confluent 3T3 cells at 37°C for 1 hr in the presence and absence of protamine sulfate (40μg/ml). The binding assays were terminated by washing with PBS.

Fig. 6b. Scatchard analysis of the data presented in Figure 5. A straight line was obtained by fitting the data using a linear least squares program. The correlation coefficient was 0.988 for the absence of protamine sulfate and 0.972 for the presence of protamine sulfate (40 μg/ml). The K_d's of 1.2×10^{-9} M and 1.6×10^{-9} M obtained for the absence and presence of protamine sulfate, respectively, were felt not to be significantly different.

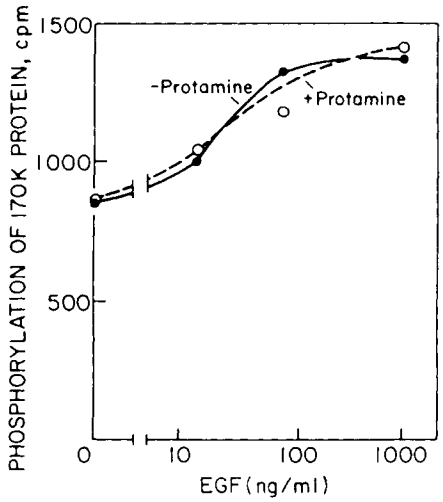


Fig. 7. Effect of protamine sulfate on EGF stimulated phosphorylation of Swiss mouse 3T3 cell membranes. The phosphorylation with [γ-^{32}P]-ATP was performed at different concentrations of EGF in the presence and absence of 100 μg/ml of protamine sulfate. The radioactivity was visualized by autoradiography using Kodak X-Omat AR films. The bands of the 170 K protein localized from the autoradiogram were excised and measured with radioactivity.

in ^{125}I-EGF binding occurred parallel to the decrease in ^{125}I-PDGF binding as protamine sulfate concentrations were increased in parallel binding assays. This increased binding of ^{125}I-EGF paralleled an increase in the number of EGF receptors measured; presumably, protamine sulfate has exposed or activated conformationally inactive EGF binding sites, because no significant change in the apparent K_d for ^{125}I-EGF binding was found. Whereas protamine sulfate influences the binding of ^{125}I-EGF to intact cells, it is without effect on the binding of ^{125}I-EGF to 3T3 cell membrane EGF receptors, and it does not influence EGF-dependent 3T3 cell membrane protein kinase activity. Thus, the effect of protamine sulfate on receptor activity may be specific in inhibiting the PDGF receptor and not the EGF receptor; the cell surface appears to be influenced secondarily to modulate EGF receptor activity. Finally, the original observations have been extended to show that certain molecular weight protamine sulfates have substantially greater activity as competitive binding proteins than does commercial protamine sulfate. Protamine sulfate is a very potent modulator of receptor activity; 400 nM protamine (fraction III) inhibited ^{125}I-PDGF binding (10 ng/ml) by 50%. Based on the competitive inhibition of ^{125}I-PDGF binding by protamine sulfate, it seems likely that protamine sulfate binds at or very near the PDGF receptor, thereby hindering sterically the access of ^{125}I-PDGF to the receptor.

The value of protamine sulfate in modulating PDGF receptor activity in complex biological systems has been established in other investigations by showing that protamine sulfate blocks the binding of PDGF to human neutrophils and monocytes [31] and the chemotactic response of neutrophils and monocytes to PDGF [2]. ^{125}I-PDGF bound to cells is displaced by protamine sulfate at times when it cannot be fully displaced by unlabelled PDGF, thus establishing the reversible and noncovalent binding of PDGF [14], as observed also when ^{125}I-PDGF was displaced by mild acid

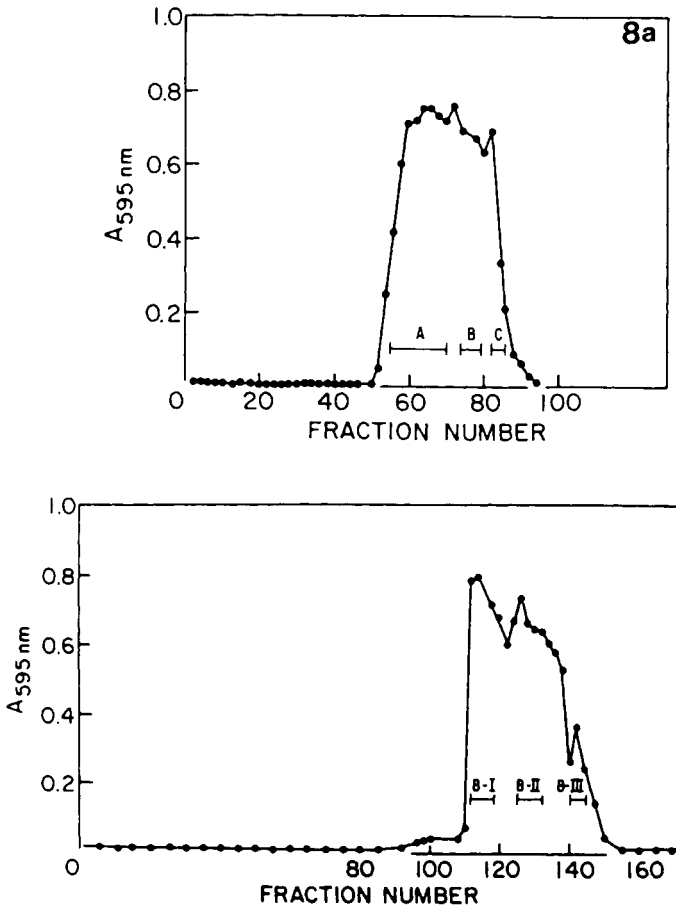


Fig. 8a. Column chromatography of protamine sulfate from salmon on a Sephadex G-50 column. Fifty mg of protamine sulfate (Sigma) was applied onto a column (1.5 × 90 cm) of Sephadex G-50 in a solvent of formic acid/acetic acid/water (25:87:888, by volume). The protein concentration was determined with Bio-Rad protein assay (optical density at 595 nm). Protamine with different molecular weights were located by SDS polyacrylamide gel electrophoresis. Fraction A contained a mixture of protamine I (MW 11,000 and protamine MW 7,800). Fraction B contained protamine III and protamine I and II. Fraction C mainly contained protamine IV (MW 3,300). The fractional volume was 1.5 ml. Ten μl of each fraction was assayed with Bio-Rad protein assay.

Fig. 8b. Rechromatography of protamine fractions obtained from the first Sephadex G-50 column chromatographs. Fraction B from the first column of Sephadex G-50 chromatographs was lyophilized and subjected to the same column (1.5 × 90 cm) of Sephadex G-50. B-I contained mainly protamine I; B-II contained protamine II; and B-III contained protamine III. The fractional volume was 0.7 ml.

treatment [32]. Protamine sulfate also competes with $p28^{v\text{-}sis}$ in conditioned media from SSV-transformed cells to block $p28^{v\text{-}sis}$ stimulated [^{3}H]-thymidine incorporation [J.S. Huang, S.S. Huang, T.F. Deuel, Cell: 39:79–81, 1984].

The precise mechanism by which protamine sulfate modulates the receptor activities of EGF is obscure. However, EGF receptors seem to be readily modulated by other ligands, such as vasopressin and phorbol esters [33,34], and by PDGF itself

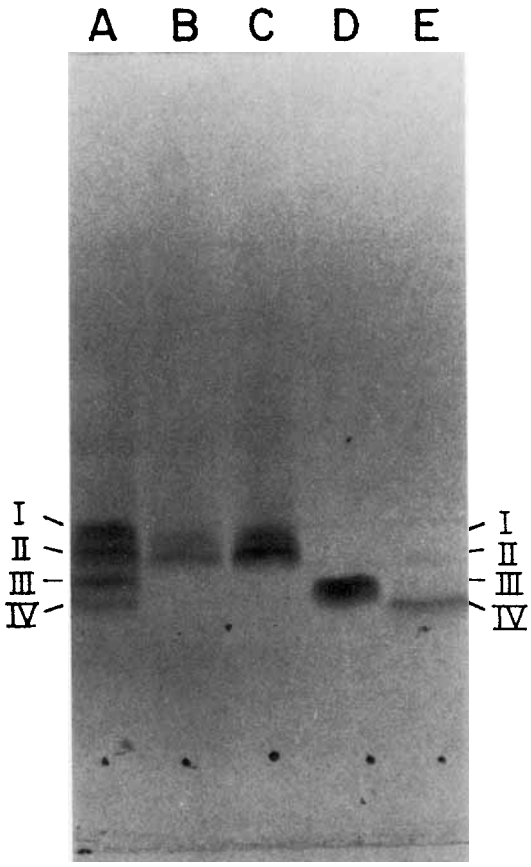


Fig. 9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified protamines. About 1-3 μg of protamines were applied. The small polypeptides (insulin B chain, apotinin, cytochrome C, and soybean trypsin inhibitor) were used as protein markers (data not shown). A) crude protamine sulfate; B) protamine I and II; C) protamine I and II; D) protamine III; E) protamine IV.

[32,35]. Phorbol tumor promoters induce EGF receptor phosphorylation [36], and the protein kinase C activated by phorbol esters phosphorylates the EGF receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity of the receptor protein [37].

PDGF and protamine sulfate share several properties in common. Both PDGF and protamine sulfate are extremely basic proteins (pI: PDGF ~ 10.2, protamine ~ 12). The amino acid compositions of both proteins are somewhat similar, with a strong predominance of basic amino acids [20,38]. Protamine stimulates a protein kinase activity that co-purifies with a DNA-binding protein of adenovirus [19] and has been shown to bind tightly to DNA and to stimulate DNA synthesis in BALB/3T3 cells [18]. ^{125}I-PDGF was tested and found to bind to DNA cellulose; ^{125}I-PDGF was retained on the column in 0.15 M NaCl but quantitatively eluted by 2 M NaCl (unpublished data). These data suggest additional similarities in the two proteins and

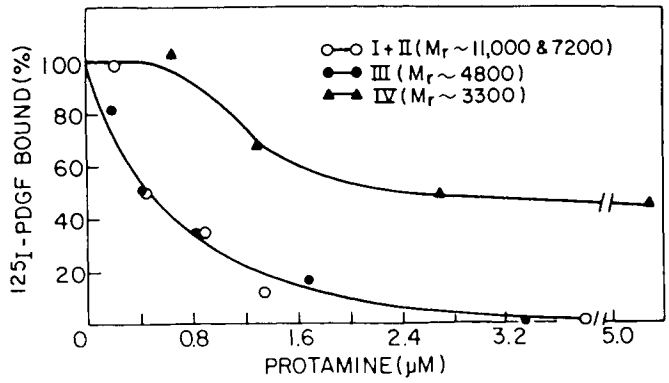


Fig. 10. Effect of purified protamines on the ^{125}I-PDGF to Swiss mouse 3T3 cells. The binding assay was performed as described in "Methods." Monolayer cell cultures were incubated with 10 ng/ml of ^{125}I-PDGF and various concentrations of protamines in 0.5 ml HEPES buffer (pH 7.4), 0.15 M NaCl containing 5% plasma-derived serum. After incubation at room temperature for 1 hr, the assays were terminated by washing with PBS. The specific binding was determined by the difference of the absence and presence of 4 μg/ml of unlabeled PDGF. The specific binding (8010 cpm) in the absence of protamine was taken as 100% binding. The preparations of protamine I + II contained equal amounts of each; the average molecular weight of this preparation was therefore assumed to be 9,000 daltons for purposes of calculations of concentrations.

support the possibility that domains of the two proteins may be sufficiently similar to result in apparent competition for the PDGF receptor.

Whereas our initial results [14] showed competition between ^{125}I-PDGF and protamine sulfate for 3T3 cell binding, the results have been extended to show competition for binding to transformed cells as well; the results also provide evidence for competition with the physiological receptor for PDGF. The present evidence thus extends the earlier preliminary findings to provide evidence that protamine sulfate may directly influence the cellular responses resulting from PDGF interactions with its physiological cell surface receptor. The work has demonstrated the specificity of the effect of protamine sulfate in competing with ^{125}I-PDGF, but not with ^{125}I-EGF, and achieved separation of crude protamine sulfate into active fractions with high inhibitory activity for ^{125}I-PDGF binding to receptors.

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REFERENCES

1. Kaplan DR, Chao FC, Stiles CD, Antoniades HN, Scher CD: Blood 53:1043–1052.
2. Deuel TF, Senior RM, Huang JS, Griffin GL: Invest 69:1046–1049, 1982.
3. Senior RM, Griffin GL, Huang JS, Walz DA, Deuel TF: J Cell Biol 96:382–385, 1983.
4. Grotendorst GR, Seppa HEJ, Kleinman HK, Martin GR: Proc. Natl Acad Sci USA 78:3669–3672.
5. Seppa H, Grotendorst GR, Seppa S, Schiffmann E, Martin GR: J Cell Biol 92:584–588, 1982.
6. Ross R, Glomset J, Kariya B, Harker L: Proc Natl Acad Sci USA 71:1207–1210, 1974.
7. Ross R, Glomset JA: New Engl J Med 295:369–377, 420–425, 1976.
8. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin A-H, Huang JS, Deuel TF: Nature 304:35–39, 1983.
9. Doolittle RF, Hunkapiller MW, Hood L, Devare SG, Robbins KC, Aaronson SA, Antoniades HN: Science 221:275–277, 1983.
10. Deuel TF, Huang JS, Huang SS, Stroobant P, Waterfield MD: Science 221:1348–1350, 1983.
11. Robbins KC, Antoniades HN, Devare SG, Hunkapiller MW, Aaronson SA: Nature 305:605–608, 1983.
12. Heldin CH, Westermark B, Wasteson A: Proc Natl Acad Sci USA 78:3663–3668, 1981.
13. Bowen-Pope DF, Ross R: J Biol Chem 257:5161–5171, 1981.
14. Huang JS, Huang SS, Kennedy BB, Deuel TF: J Biol Chem 257:8130–8136, 1982.
15. Williams LT, Tremble P, Antoniades HN: Proc Natl Acad Sci USA 79:5867–5870, 1982.
16. Huang SS, Huang JS, Deuel TF: "Cold Spring Harbor Conferences on Cell Proliferation and Cancer: The Cancer Cell," Vol I, pp 43–49, 1984.
17. Taylor S, Folkman J: Nature 297:307–312, 1982.
18. Yoshitake Y, Nishikawa K, and Adachi K: Cell Struct Funct 7:229–243, 1981.
19. Cajean-Feroldi C, Loeb J, Meguenni S, Girard M: Eur J Biochem 120:79–87, 1981.
20. Deuel TF, Huang JS, Proffitt RT, Baenziger JU, Chang D, Kennedy BB: J Biol Chem 256:8896–8899, 1981.
21. Nishimura J, Huang JS, Deuel TF: Proc Natl Acad Sci USA 79:4303–4307, 1982.
22. Comens PG, Simmer RL, Baker JB: J Biol Chem 257:42–45, 1982.
23. Bradford MM: Anal Biochem 72:248–254, 1976.
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
25. Laemmli UK: Nature 227:680–685, 1970.
26. Huang JS, Proffitt RT, Baenziger JU, Chang D, Kennedy BB, Deuel TF: Marchesi V et al (eds): "Differentiation and Function of Hematopoietic Cell Surfaces." New York: Alan R. Liss Inc, pp 225–230.
27. Bowen-Pope DF, Vogel A, Ross R: Proc Natl Acad Sci USA 81:2396–2400, 1984.
28. Ek B, Westermark B, Wasteson A, Heldin C-H: Nature 295:419–420, 1982.
29. Wrann MM, Fox CF: J Biol Chem 254:8083–8086.
30. Cohen S, Ushiro H, Stoscheck C, Chinkers M: J Biol Chem 257:1523–1531, 1982.
31. Kimura A, Huang JS, Deuel TF: Fed Proc 42:1830, 1983.
32. Bowen-Pope DF, Dicorleto PE, Ross R: J Cell Biol 96:679–683, 1983.
33. Rozengurt E, Brown KD, Pettican P: J Biol Chem 256:716–722, 1981.
34. Lee LS, Weinstein JB: Science 202:313–315, 1978.
35. Collins MKL, Sinnett-Smith JW, Rozengurt E: J Biol Chem 258:11689–11693, 1983.
36. Iwashita S, Fox CF: J Biol Chem 259:2559–2567, 1984.
37. Cochet C, Gill GN, Meisenhelder J, Cooper JA, Hunter T: J Biol Chem 259:2553–2558, 1984.
38. Ando T, Yamasaki M, Suzuki K: Mol Biol Biochem Biophys 12:16–26, 1973.