Articles

Identification and Characterization of a Cytosolic Protein Tyrosine Kinase of HeLa Cells†

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Received July 17, 1991; Revised Manuscript Received December 31, 1991

ABSTRACT: Fractionation of a cytosolic extract of HeLa cells revealed the existence of a highly active protein tyrosine kinase. Chromatographic fractionation of the extract resulted in partial purification of a single enzymatic activity that coeluted with a 94-kDa polypeptide. In vitro phosphorylation of the isolated enzyme showed that p94 was the only polypeptide phosphorylated and only the tyrosine residue(s) was (were) modified. The fractionated enzyme (p94 kinase) also phosphorylated a number of other nonspecific substrates exclusively on tyrosine residues. Unlike other protein tyrosine kinases that have been characterized, p94 kinase is relatively insensitive to inhibition by the isoflavone genistein. Using two different antisera, we provided evidence that the HeLa p94 kinase is most likely the FER gene product, which was previously shown to be expressed in a wide variety of cell types. These results represent the first biochemical characterization of the cellular FER gene product and also provide a basis for studying the biochemistry of tyrosine kinase function in HeLa cells.

Protein tyrosine kinases (PTKs)[1] have been a subject of extensive investigation because of their involvement in oncogenesis, control of cell growth, and development in all eukaryotic cells (Hunter & Cooper, 1985; Hunter, 1987; Bosler & Hafen, 1988; Ullrich & Schlesinger, 1990; Morla et al., 1989; Gould & Nurse, 1989). A large number of protein tyrosine kinases have been identified, and they can be broadly categorized into two groups according to their subcellular localization: receptor and cytoplasmic kinases. Receptors of epidermal growth factor and platelet-derived growth factor are representatives of the first type and are distinguishable by the presence of a transmembrane sequence in these polypeptides. Examples of cytoplasmic kinases include those encoded by c-*src*, c-*fes*, and c-*abl* genes. Although these latter kinases do not possess membrane-spanning sequences, some of them have been shown to associate with plasma membrane through interaction with fatty acids covalently attached to the polypeptides (Marchildon et al., 1984; Buss & Sefton, 1985). Members of each of these two groups can be further subclassified according to sequence homology. Receptor tyrosine kinases have been shown to have an important function in transduction of signals elicited by the ligands [reviewed in Ullrich and Schlessinger (1990)]. The function of the cytoplasmic kinases has not yet been determined. The existence of multiple cytoplasmic kinases suggests that some of these enzymes may have cell-type-specific functions. For instance, $p56^{lck}$ is expressed primarily in T-lymphocytes and has been shown to interact with CD4 and CD8 proteins of T-cells (Rudd et al., 1988; Veillette et al., 1988). However, it has not yet been possible to demonstrate such functional correlation for most of the other cytoplasmic enzymes. It is also intriguing that in some cells and tissues more than one type of cytoplasmic PTKs are expressed. The basis for this multiplicity has been difficult to understand because the in vitro properties of almost all of these cytoplasmic enzymes have proven to be quite similar. Clearly, a better understanding of the biochemical properties of the cytoplasmic kinases is required before their biological functions can be elucidated. It is our goal to characterize biochemical properties of the cytoplasmic tyrosine protein kinases in order to be able to identify their cellular functions. In this report, we describe the identification and biochemical characterization of a soluble protein tyrosine kinase from HeLa cells.

Materials and Methods

Materials. [Val^5]angiotensin II, sodium orthovanadate, enolase, and poly(Glu,Tyr) (4:1) were obtained from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear Inc. Phosphocellulose paper (P81) was from Whatman. Heparin–Sepharose CL-6B, Superose 6, Mono Q HR5/5, Mono S HR5/5, and protein A–Sepharose were from Pharmacia. Genistein and rabbit anti-rat IgG was from ICN Biochemicals. Tissue culture media and sera were purchased from Sigma. Peptide analogues of $p34^{cdc2}$ were synthesized on a Milligen Excel synthesizer using Fmoc protecting groups. The synthetic peptides were purified by C_{18} reverse-phase chromatography. An expression plasmid that encodes a fusion of bacterial TrpE and amino acids 502–675 of the human FER gene product was provided by Dr. Tony Pawson. This segment of the FER gene product encodes a portion of the SH2 domain and part of the ATP binding site (Hao et al., 1989). The fusion protein was isolated as inclusion bodies from *Escherichia coli*, gel-purified, and used to immunize New Zealand White rabbits. Immune antisera were adsorbed with TrpE protein before use in western blot analyses. The antisera do not

† This research was supported by a grant from the New Jersey Commission for Cancer Research.

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[1] Abbreviations: PTK, protein tyrosine kinase; kDa, kilodalton(s); SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH2, *src* homology 2.

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cross-react with other SH2-containing PTKs such as pp60^{src} (data not shown).

Cell Culture. HeLa S3 cells were grown in suspension in Joklik modified Eagle's medium (MEM) supplemented with 5% (v/v) calf serum. Cells were harvested at a density of (5–10) × 10^5 cells/mL. U937 cells (human histiocytic lymphoma) were obtained from Dr. A. Rashidbaigi of Robert Wood Johnson Medical School and were cultured in RPMI 1640 supplemented with 10% fetal calf serum. A431 cells were cultured in Dulbecco's MEM plus 10% calf serum.

Enzyme Purification. All purification steps were carried out at 4 °C unless otherwise indicated. HeLa cells were pelleted by centrifugation at 700*g* for 10 min and were resuspended in hypotonic lysis buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, and 5 mM $MgCl_2$) at about 10^8 cells/mL. The cell suspension was incubated for 10 min on ice and homogenized with 10–15 strokes in a Dounce homogenizer with a tight-fitting pestle, until more than 90% of the cells were disrupted. After the addition of 1 volume of buffer containing 0.5 M sucrose, 20 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, and 0.4 mM EDTA, the homogenate was centrifuged for 10 min at 12000*g* to remove nuclei and mitochondria and was centrifuged again at 150000*g* for 1 h. The supernatant (S_{150}) was saved and was made 20 mM in EDTA. The lysate was applied to a 30-mL column of phosphocellulose (P11) that had been equilibrated in buffer A (20 mM Tris·HCl, pH 8, 10% glycerol, and 0.2 mM EDTA) containing 0.15 M NaCl. The column was washed with the same buffer, and bound proteins were eluted with a linear gradient (80 mL × 80 mL) of 0.15–0.5 M NaCl in buffer A. Fractions that contained activity were pooled and were diluted with 3 volumes of buffer A. The diluted material was applied to a 10-mL column of heparin–Sepharose equilibrated in buffer A containing 0.1 M NaCl. Kinase activity was eluted with a linear gradient (40 mL × 40 mL) of 0.1–0.9 M NaCl in buffer A. Active fractions were pooled and diluted with 4 volumes of buffer A. The material was then applied to an FPLC Mono Q column that had been equilibrated in buffer A containing 0.15 M NaCl. Kinase activity was eluted with a 30-mL linear gradient of 0.15–0.5 M NaCl in buffer A. Fractions that contained kinase activity were pooled and diluted with 2 volumes of buffer A. The fraction was then applied to an FPLC Mono S column in buffer A containing 50 mM NaCl. Kinase activity was eluted with a 25-mL linear gradient of 50–400 mM NaCl in buffer A. Active fractions were quick-frozen in liquid nitrogen and stored at −80 °C.

Peptide and Protein Phosphorylation Assays. PTK activity was determined using [Val^5]angiotensin II as substrate as described previously, but with some modifications (Wong & Goldberg, 1984). Reaction mixtures were incubated at 26 °C for 1 h in a total volume of 25 μL, containing 50 mM Tris·HCl, pH 7.7, BSA at 1.2 mg/mL, 1 mM peptide, 10 mM $MnCl_2$, 2 μM [γ-^{32}P]ATP (specific activity 60 Ci/mmol), 100 μM, Na_3VO_4, and 14 mM 2-mercaptoethanol. In some reactions that employed purified kinase preparations that had been dialyzed, NaCl was added to the reaction mixtures to a final concentration of 200 mM. Reaction was stopped by transferring the reaction mixtures to an ice–water bath and addition of 45 μL of cold 5% (w/v) trichloroacetic acid containing 3.5 mM ATP. The inclusion of ATP in the stop buffer reduced background incorporation to less than 1000 cpm. The mixtures were incubated for 5 min on ice and were centrifuged in a microfuge at 4 °C for 5 min. Aliquots (35 μL) of the supernatants were spotted onto P81 ion-exchange chromatography paper squares (2 cm^2). The filters were washed 6 times, 5 min each, in 0.425% phosphoric acid (10 mL/square) and once with acetone. They were dried and counted in a liquid scintillation counter. One unit of PTK activity is defined as the activity that phosphorylates 1 pmol of [Val^5]angiotensin II in 1 h under the conditions described above. Data from kinetic experiments were subjected to curve fitting by a least squares regression method.

Autophosphorylation assays were carried out in a total volume of 50 μL in the presence of 50 mM Tris·HCl, pH 7.7, 10 mM $MnCl_2$, 200 mM NaCl, 14 mM 2-mercaptoethanol, and 0.1 μM [γ-^{32}P]ATP (3000 Ci/mmol). Reaction mixtures were incubated at 26 °C for 30 min. Reactions were terminated by addition of 500 μL of 0.1% SDS containing 20 μg of cytochrome *c*. Proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 5%. Precipitated proteins were pelleted by centrifugation, rinsed with cold acetone, and solubilized by boiling in SDS–PAGE sample buffer. Phosphorylation of enolase was assayed as above except that denatured enolase was used at a final concentration of 0.2 mg/mL and [γ-^{32}P]ATP concentration was 40 nM (3000 Ci/mmol). Rabbit muscle enolase was denatured with acetic acid as described (Cooper et al., 1984).

In vitro phosphorylation of the random copolymer peptide poly(Glu,Tyr) was carried out as described above for phosphorylation of enolase. The polypeptide substrate was used in a final concentration of 0.2 or 2.0 mg/mL. Reaction was stopped by placing the reaction mixtures on ice and adding 10 μL of 3.5 mM ATP to terminate the incorporation of radioactive ATP. The entire reaction mixtures were spotted onto P81 squares, which were dried and then incubated in cold 5% trichloroacetic acid for 15 min without shaking. The filters were washed 6 times for 5 min in cold 5% trichloroacetic acid on a shaker (10 mL/square). They were then rinsed with acetone, dried, and counted.

Immune Complex Kinase Assays. Rabbit antiserum against the synthetic dodecapeptide Lys-Gln-Ile-Pro-Val-Lys-Trp-Thr-Ala-Pro-Glu-Ala [corresponding to amino acids 1080–1091 of the Fujinami sarcoma virus (FSV) transforming protein p140] and rat FSV-specific regressing-tumor antiserum (anti-FST) were gifts from Dr. Ricardo A. Feldman, National Cancer Institute (Feldman et al., 1986). Whole-cell extracts were prepared from HeLa cells and U937 cells by lysing them in cold buffer containing 10% glycerol, 20 mM Tris·HCl, pH 7.5, 0.2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 1% Triton X-100. The lysates were clarified by centrifugation for 10 min at 10000*g* at 4 °C, and the supernatant was stored at −80 °C until further analyses. Immunoprecipitation was carried out as described previously (Wong & Goldberg, 1984). Immune complexes were washed three times with 1 mL of buffer B (10% glycerol, 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) and two times with buffer C (10% glycerol, 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl, and 0.1% Tween 20). The complexes were resuspended in a total volume of 40 μL of 50 mM Tris·HCl, pH 7.7, 10 mM $MnCl_2$, 200 mM NaCl, 14 mM 2-mercaptoethanol, and 50 nM [γ-^{32}P]ATP (3000 Ci/mmol) and were incubated for 10 min at 20 °C. Beads were washed twice with buffer B, suspended in 40 μL of SDS–PAGE sample buffer, and incubated for 3 min at 90 °C. After separation on a 7.5% SDS-containing polyacrylamide gel, radiolabeled proteins were detected by autoradiography. For subcellular localization of p94 kinase activities, cells were disrupted and S_{150} fraction was prepared as described above. The pellet from the 150000*g* centrifugation was resuspended

by homogenization in 100 mL of wash buffer (25 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.25 mM EDTA, and 14 mM 2-mercaptoethanol). The suspension was centrifuged at 150000*g* again, and the supernatant was discarded. The pellet was resuspended again by homogenization in 16 mL of wash buffer. Triton X-100 was added to a final concentration of 1% (v/v), and the mixture was allowed to stand on ice for 5 min. The mixture was then centrifuged at 250000*g* for 45 min to remove insoluble materials. The supernatant, which contained solubilized membrane proteins, was saved and used in immune complex kinase assays. A membrane fraction was prepared from A431 cells using a similar procedure.

Western Blotting Analyses. Protein samples were fractioned by SDS–PAGE, and the separated polypeptides were transferred electrophoretically to a piece of Immobilon-P filter (Millipore). The filter was incubated for 1 h in blocking buffer (5% dry milk, 10% calf serum, 20 mM Tris·HCl, pH 7.4, 0.1 M NaCl, 0.1% Tween 20, and 0.2 mM EDTA). Antiserum to TrpE-FER fusion protein was diluted 1:1000 in blocking buffer and was added to the filter. After 1 h, the filter was washed three times with TBST (20 mM Tris·HCl, pH 7.4, 0.1 M NaCl, 0.1% Tween 20, and 0.2 mM EDTA). The filter was then incubated for 1 h in TBST containing peroxidase-conjugated goat anti-rabbit IgG at 1:5000 dilution. The filter was then washed three times with TBST and once with saline. Antibody binding was detected using the Amersham Enhanced Chemiluminescence detection kit.

Other Methods. Protein concentrations were assayed as described by Bradford using bovine albumin as a standard (Bradford, 1976). Polyacrylamide gels were stained with silver nitrate as described. For phosphoamino acid analyses, phosphorylated polypeptides were extracted from a dried gel and were subjected to acid hydrolysis as described previously. The hydrolysates were analyzed by one-dimensional electrophoresis at pH 3.5 (0.5% pyridine, 5% acetic acid, and 5 mM EDTA) at 500 V for 1.5 h on thin-layer cellulose plates (0.1 mm thick). Phosphoamino acid standards were detected by spraying with ninhydrin, and phosphorylated residues were detected by autoradiography. Identification of phosphorylated amino acids in the $p34^{cdc2}$ peptide analogues was carried out as described previously (Sullivan & Wong, 1991).

Results

Identification of the Major HeLa Protein Tyrosine Kinase Activity. As part of our effort to characterize the biochemical properties of cytosolic protein tyrosine kinases, we fractionated a cytosolic fraction of HeLa cells and assayed for peptide kinase activity. We chose to examine HeLa cells, primarily because these cells have proven to be an excellent source of materials for studying biochemical functions of eukaryotic enzymes and yet here had not been any systematic description of their PTK activities. We prepared a high-speed supernatant from a HeLa cell homogenate and assayed for PTK activity using [Val^5]angiotensin II as a substrate. The unfractionated cell lysate had no detectable kinase activity even in the presence of sodium vanadate and ammonium molybdate, which inhibit tyrosine-specific phosphatases. Fractionation of the lysate on phosphocellulose led to the recovery of a highly active fraction of enzyme activity that phosphorylated the peptide substrate. The enzyme was fractionated further on heparin–Sepharose, Mono Q, and Mono S columns, and the elution profiles are shown in Figures 1 and 2. The enzyme was recovered in a single peak from the Mono S column, and kinase activity coeluted with a 94-kDa polypeptide (Figure 2). Although other polypeptides were also present in these fractions, they clearly did not cofractionate precisely with kinase activity. We will henceforth refer to the HeLa kinase activity as p94 kinase. Results of the fractionation procedures are summarized in Table I.

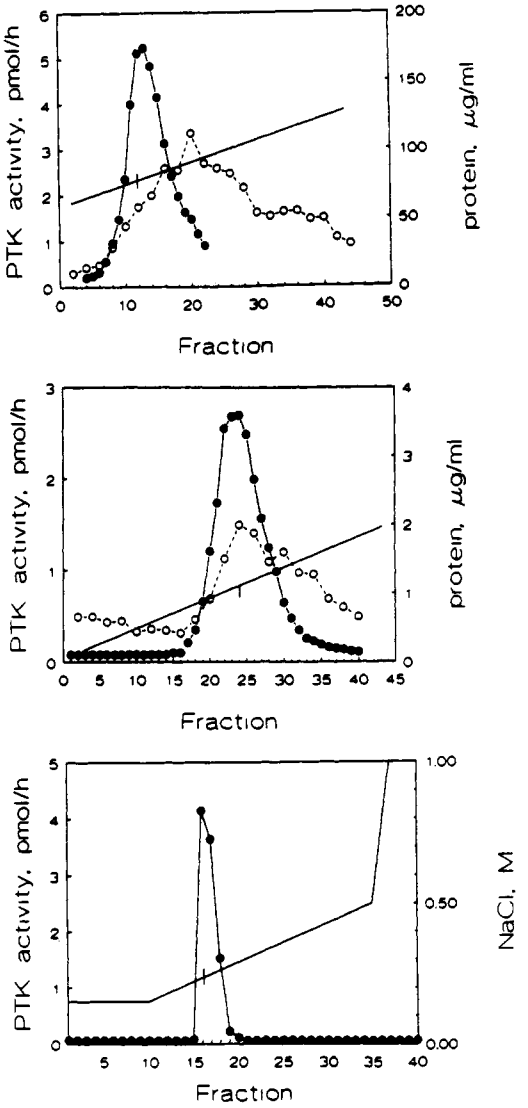
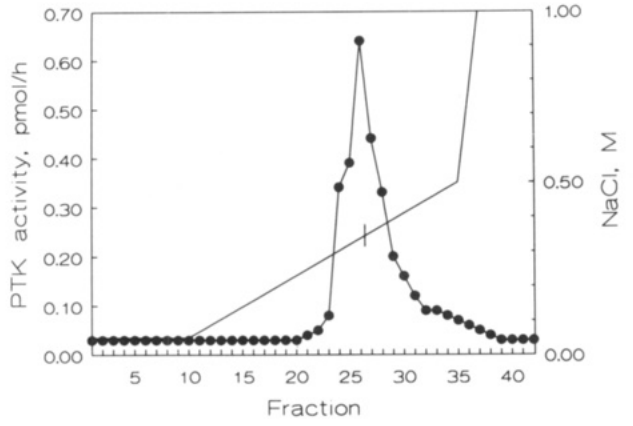


FIGURE 1: Fractionation of HeLa protein tyrosine kinase. An S_{150} fraction of a HeLa cell lysate was fractionated by ion-exchange chromatography. Solid lines in each panel indicate where salt gradient was applied to elute bound proteins. In all cases, no significant amount of enzyme activity was recovered in flow-through fractions. Fractions were assayed for protein concentrations (open circles) and/or kinase activity (filled circles). In kinase assays, 1 μL of each column fraction was used. (Top panel) Elution profile on a phosphocellulose column. Gradient was from 0.15 to 0.5 M NaCl. (Middle panel) Elution profile on heparin–Sepharose column. Gradient was from 0.1 to 0.9 M NaCl in buffer A. (Bottom panel) Elution profile on a Mono Q column. Gradient was from 0.15 to 0.5 M NaCl in buffer A.

Characterization of the Purified Enzyme. Using angiotensin as substrate, we determined optimal reaction conditions for the purified HeLa kinase. The enzyme exhibited optimal kinase activity when assayed at 26 °C at pH between 7.6 and 7.8. The enzyme showed an exclusive preference for Mn^{2+} and was totally inactive with Mg^{2+} or Ca^{2+}. Phosphorylation of angiotensin was linear for at least 2 h (data not shown). We determined kinetics of phosphorylation of [Val^5]angiotensin II using purified p94 kinase. With peptide or ATP as varying substrates, linear double-reciprocal plots that intersect were obtained. Slopes and y-intercepts were replotted for derivation of kinetic constants (Segel, 1975). From the secondary plots, we obtained K_m values of 7.1 μM and 0.36 mM for ATP and [Val^5]angiotensin II, respectively. In addition,



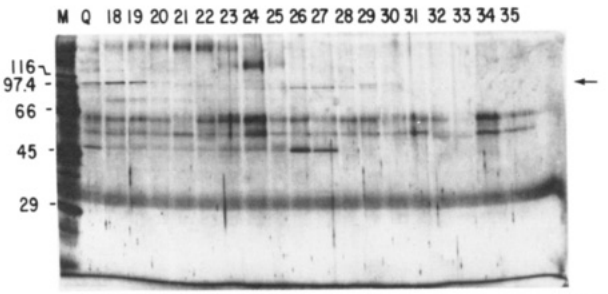


FIGURE 2: Elution profile of HeLa protein tyrosine kinase on a Mono S column. Active fractions recovered from the Mono Q column (Figure 1) were pooled, and NaCl concentration was adjusted to 50 mM by diluting with buffer A. The material was applied to a Mono S column, and bound proteins were eluted with a gradient of 50–400 mM NaCl in buffer A. Fractions were assayed for peptide kinase activity (filled circles). Top panel shows elution profile of PTK activity. Bottom panel shows polypeptide compositions of column fractions from the Mono S column. Numbers on top of the panel indicate gradient fractions from the column. Proteins in lane M are molecular weight markers, and those in lane Q are proteins applied to the Mono S column. Numbers on the left side show the molecular mass of marker proteins in kDa. Arrow on right side points to the 94-kDa polypeptide that coeluted with peptide kinase activity.

Table I: Recovery of Proteins and Enzymatic Activity during Fractionation of HeLa Protein Tyrosine Kinase[a]

fraction	protein (mg)	PTK activity (units)	specific activity (units/mg)
(I) S_{150}	1500	8530[b] (100)	5.69[b]
(II) phosphocellulose	3.41	8530 (100)	2 500
(III) heparin–Sepharose	0.362	1920 (23)	5 300
(IV) Mono Q	0.025	1210 (14)	48 400
(V) Mono S	0.003	485 (6)	162 000

[a] Data shown were obtained from an S_{150} fraction prepared from 20 L of HeLa S3 cells. PTK activity was measured using [Val^5]angiotensin II as substrate. Numbers in parentheses show recovery of kinase activity expressed as a percentage of that of fraction I. [b] Amount of enzyme activity and specific activity in the S_{150} fraction were estimated from data obtained for the phosphocellulose fraction. This was done because no kinase activity was detectable in fraction I, presumably as a result of the presence of inhibitors.

K_{ia}(ATP) and V_{max} were determined to be 20 μM and 170 nmol/(min·mg), respectively. In addition to [Val^5]angiotensin II, the purified HeLa PTK also phosphorylates a random copolymer of glutamic acid and tyrosine (Figure 3A). Poly(Glu,Tyr) was previously shown to be phosphorylated by a variety of protein tyrosine kinases (Braun et al., 1984). Like all other known protein tyrosine kinases, the HeLa enzyme phosphorylates enolase in vitro (Cooper et al., 1984) (Figure 3B). In protein phosphorylation assays, phoshorylation of p94 was also detected (Figure 3B, lane 1). Phosphoamino acid

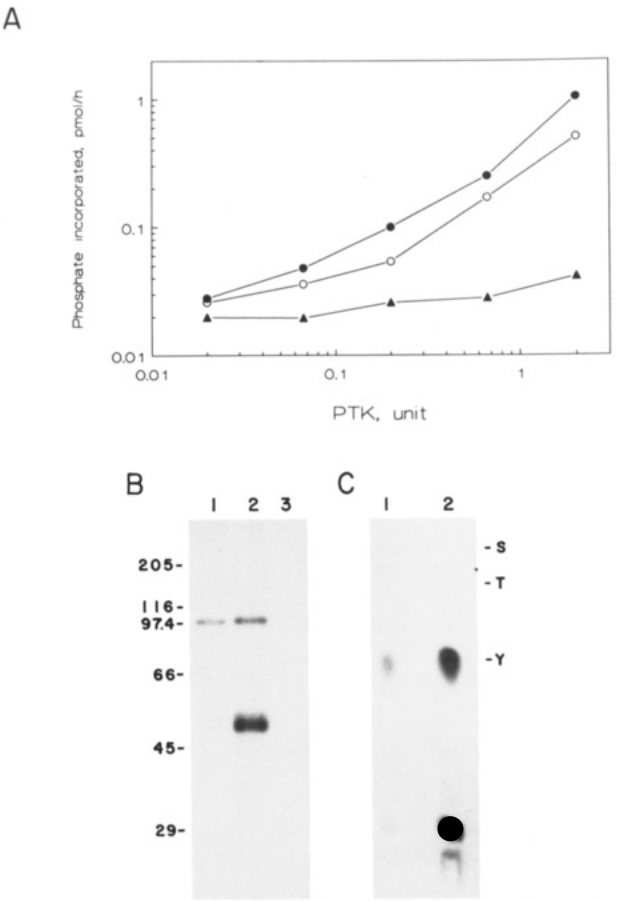


FIGURE 3: Substrate specificity of purified HeLa protein tyrosine kinase. (A) A random copolymer poly(Glu,Tyr) was tested as an in vitro substrate for HeLa p94 kinase (Mono S fraction). Increasing amounts of units of PTK activity, as defined in Materials and Methods, were used. Results shown are amounts of ^{32}P incorporated into acid-insoluble materials using enzyme alone (triangles), or with enzyme in the presence of poly(Glu,Tyr) at 0.2 mg/mL (open circles) and at 2 mg/mL (filled circles). (B) Acid-denatured enolase was assayed for its ability to be phosphorylated by purified HeLa PTK kinase. Lane 1: HeLa PTK alone (approximately 20 ng). Lane 2: HeLa PTK and enolase at 0.2 mg/mL. Lane 3: Enolase alone. Numbers on the left side are sizes of molecular weight markers in kDa. Exposure was for 2 h with an intensifying screen. (C) Phosphoamino acid analyses of products of phosphorylation shown in panel B. Phosphorylated p94 and enolase were recovered from the gel and were subjected to partial acid hydrolysis. The hydrolysates were separated by thin-layer electrophoresis at pH 3.5. Lane 1: p94. Lane 2: Enolase. Letters on the right side indicate the locations of phosphoamino acid standards (S = phosphoserine, T = phosphothreonine, and Y = phosphotyrosine) that were detected by ninhydrin staining.

analyses of the phosphorylated 94-kDa polypeptide and enolase showed that both polypeptides were phosphorylated exclusively on tyrosine residues (Figure 3C). We also examined if p94 kinase was able to phosphorylate a peptide analogue of the cell cycle regulator, p34^{cdc2}. The peptide contained two tyrosine residues, one of which (residue 10) corresponds to the site shown to be phosphorylated in vivo (Tyr15) (Draetta et al., 1988; Gould & Nurse, 1989). Peptide cdcI was found to be phosphorylated by HeLa p94 kinase, with kinetic constants that are slightly more favorable than those for [Val^5]angiotensin II (Table II). Peptide cdcII, which contained a Tyr → Phe substitution at residue 14, was comparable to cdcI in its substrate properties. In contrast, the same substitution at residue 10 resulted in a significantly less efficient substrate in cdcIII. Phosphoamino acid analyses of the phosphorylated cdc peptide showed that only tyrosine was phosphorylated (data not shown). Since the peptide contains two tyrosine residues, it was necessary to determine which of the two

Table II: Apparent Kinetic Constants for Phosphorylation of cdc Peptides and [Val^5]angiotensin II Using HeLa p94 Kinase[a]

peptide	sequence	K_m (mM)	V_{max} [nmol/(min·mg)]
[Val^5]angiotensin II	DRVYVHPF	0.40	110
cdcI	KIEKIGEGTYGVVYK	0.27	350
cdcII	KIEKIGEGTYGVVFK	1.10	242
cdcIII	KIEKIGEGTFGVVYK	1.23	56

[a] Apparent K_m and V_{max} were obtained from intercepts of double-reciprocal replots of kinetic data, which where measured using 10 μM ATP.

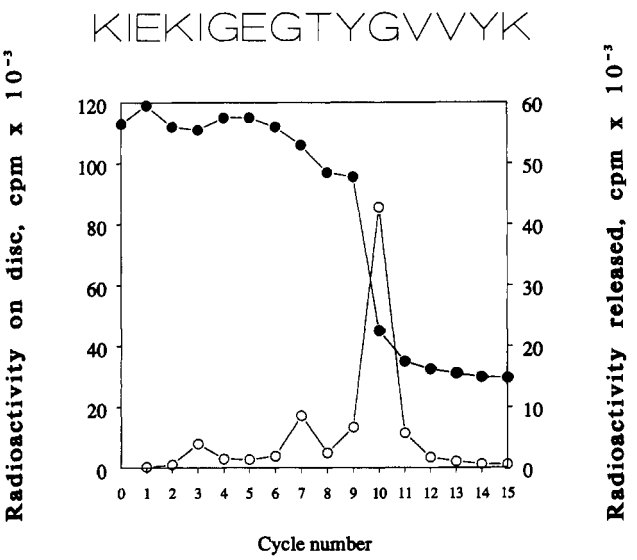


FIGURE 4: Identification of phosphorylation site in peptide cdcI. Products of a kinase assay using peptide cdcI and HeLa p94 kinase were freed of ATP and isolated. The phosphopeptide was immobilized on an arylamine disk and subjected to manual Edman degradation. At the end of each cycle, the trifluoroacetic acid used in the cleavage reaction was saved and combined with a subsequent TFA/phosphoric acid wash. The combined liquid phase was counted in a scintillation counter to determine the amount of phosphate released (right y-axis, open circles). The washed disk was also counted to measure the amount of radioactivity that remained on the filter (left y-axis, filled circles).

tyrosine residues was modified. The phosphopeptide was isolated and was subjected to Edman degradation. The bulk of the radioactivity was released from the immobilized peptide at cycle 10, and no significant release of phosphate was detected at cycle 14 (Figure 4).

Recently, a number of reagents have been shown to specifically inhibit protein tyrosine kinases. For instance, the isoflavone genistein and related analogues have been shown to inhibit a number of different protein tyrosine kinases at concentrations that have little or no effect on serine or threonine kinases (Akiyama et al., 1987). We examined the effect of genistein on the activity of purified p94 kinase. The EGF receptor kinase activity, which is highly enriched in a membrane fraction prepared from A431 cells, was inhibited by the addition of genistein (Figure 5, open circles). In agreement with results previously reported, a concentration of about 10 μg/mL genistein was sufficient to achieve 50% inhibition of receptor kinase activity (Akiyama et al., 1987). In contrast, p94 kinase activity was relatively insensitive to genistein at concentrations up to 80 μg/mL (Figure 5, closed circles). At low concentrations of genistein, a slight stimulation of p94 kinase was consistently observed. The basis of this stimulation is presently unknown.

Identification of p94 Kinase. In order to determine if the HeLa p94 kinase is identical to any of the other known cytoplasmic kinases, we carried out immune complex kinase

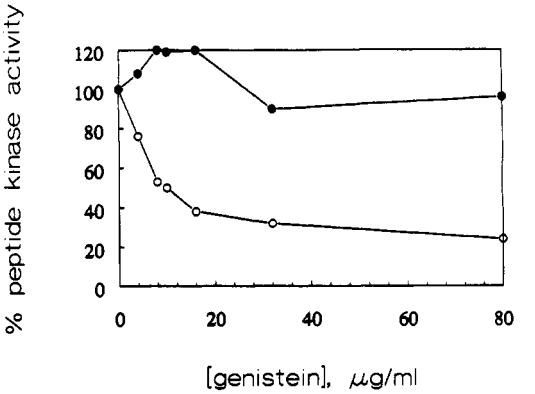


FIGURE 5: Effect of genistein on peptide kinase activity of purified HeLa PTK and A431 membrane kinase. A431 cell membrane (2 μg) (open circles) or purified p94 kinase (10 ng) (filled circles) was used in kinase assays. Effect of genistein on kinase activity is expressed as percentage of activity obtained in the presence of vehicle, i.e., 4% dimethyl sulfoxide alone. Dimethyl sulfoxide alone results in a 100% stimulation of the A431 membrane activity and a 20% inhibition of p94 kinase activity. For the two enzymatic activities, 100% was 10 pmol (A431) and 9 pmol (HeLa) of phosphate incorporated per reaction in 15 min and 1 h, respectively.

assays using an anti-peptide serum that recognizes the sequence KQIPVKWTAPEA. The sequence is part of the v-*fps* protein (p140) and has been shown to be one of the conserved domains that are hallmarks of sequences that encode tyrosine kinases (Shibuya & Hanafusa, 1982; Hanks et al., 1988). The antiserum was previously used to identify NCP92, or p92$^{c\text{-}fps}$, which is encoded by the cellular *fps* oncogene and which is expressed predominantly in cells of myeloid lineage (Feldman et al., 1986). The serum was found to react also with a 94-kDa polypeptide (NCP94) that is expressed in many animal tissues. Immune complex kinase assay of a HeLa whole-cell lysate with the anti-peptide serum resulted in phosphorylation of a 94-kDa polypeptide (Figure 6, lane 1). A small amount of the same polypeptide was also immunoprecipitated with an anti-FST antibody (lane 2), which was directed against the v-*fps* protein (Feldman et al., 1986). In addition to p94, a number of other polypeptides were also phosphorylated. However, these other proteins were also present in immunoprecipitates obtained with a nonimmune rabbit antibody (lane 3) and are probably results of nonspecific interactions. As a control to illustrate specificity of the two antisera used, we performed also immune complex kinase assays using a whole-cell lysate prepared from U937 cells. As Feldman et al. showed, both NCP92 and NCP94 are expressed in U937 cells and can be immunoprecipitated with the anti-peptide serum, because the peptide sequence is present in both polypeptides (Feldman et al., 1986). In contrast, only NCP92 was immunoprecipitated by anti-FST, which is specific for the v-*fps* protein. The anti-FST serum immunoprecipitated a small amount of p94 from HeLa cells, but not from U937 cells, simply because of the greater abundance of the protein in HeLa cells. The anti-peptide serum was used also to immunoprecipitate the kinase activity that we have purified from HeLa cells. Although the background due to nonspecific interactions was rather substantial when purified enzyme was used, it is apparent that the anti-peptide antibodies also specifically recognized the purified enzyme (Figure 6, compare lanes 7 and 9). The use of purified enzyme in these assays usually results in poor recovery of activity in the immune complex, possibly because the purified enzyme cannot withstand the prolonged manipulations and incubations involved. However, it should be emphasized that supernatants from such immunoprecipitations were consistently devoid of kinase activity. These results suggest that the kinase

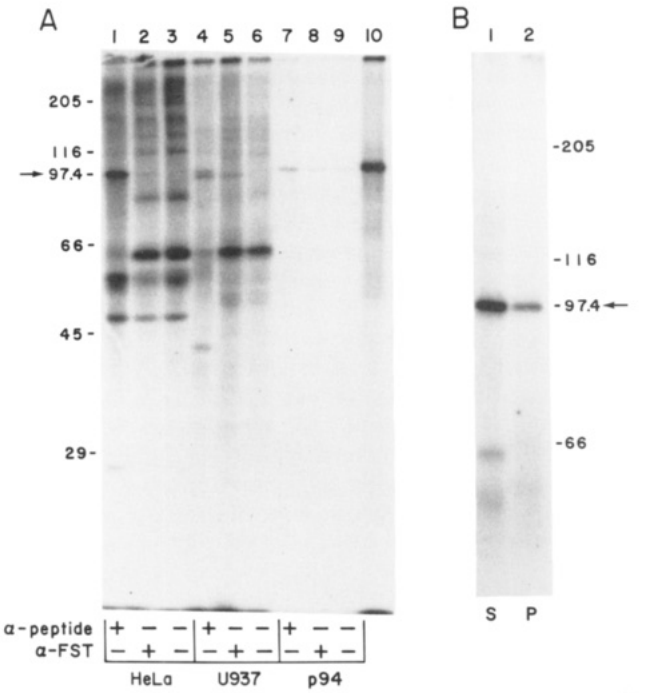


FIGURE 6: Immune complex kinase assays of purified HeLa PTK and lysates prepared from HeLa and U937 cells. (A) Mono S fraction of p94 kinase and whole-cell lysates were immunoprecipitated with anti-peptide serum (lanes 1, 4, and 7), anti-FST serum (lanes 2, 5, and 8), or rabbit anti-rat IgG (lanes 3, 6, and 9). In lanes 1–3 are shown results obtained with HeLa cell lysate (40 μg per incubation), and in lanes 4–6 are those obtained with U937 cell lysate (200 μg per incubation). Lanes 7–10 show results of immune complex kinase assays obtained with approximately 50 ng of purified HeLa p94 kinase supplemented with 20 μg of bovine albumin. In lane 10 is shown the result of autophosphorylation of the purified kinase preparation. (B) Subcellular localization of HeLa p94 kinase. Equal amounts (200 μg) of cytosolic (lane 1) and membrane (lane 2) proteins were analyzed in the immune complex kinase assay using anti-peptide serum. In both panels A and B, numbers on the side are sizes of molecular mass markers in kDa. Arrows point to 94-kDa polypeptide that was immunoprecipitated by anti-peptide serum. Exposure was for 14 h (panel A) or 2.5 h (panel B) without an intensifying screen.

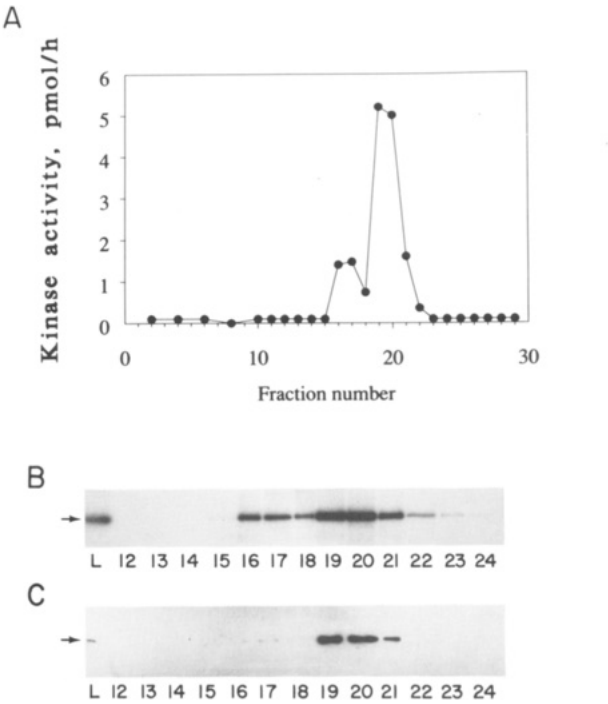


FIGURE 7: Elution profile of HeLa protein tyrosine kinase on second Mono Q column. Active fractions from the Mono S column of Figure 2 were pooled and applied to a second Mono Q column. Enzyme was eluted with a linear gradient (20 mL) of 0.15–0.5 M NaCl (fractions 11–30). (Panel A) Fractions were collected and 15-μL aliquots were assayed for peptide kinase activity (filled circles). (Panel B) Polypeptide compositions of column fractions were analyzed by SDS–PAGE and silver staining. (Panel C) Protein kinase activity in column fractions was assayed, and phosphorylated proteins were analyzed by SDS–PAGE and autoradiography. In lanes marked L are shown polypeptides that were applied to the column. Arrows on the left side point to where p94 migrated.

activity we have purified from HeLa cells is very similar to that of NCP94. We also examined if p94 kinase of HeLa cells was found exclusively in the soluble fraction of a cell homogenate. Cytosolic and solubilized membrane fractions were prepared and were analyzed by the immune complex kinase assay using anti-peptide serum. As shown in Figure 6 (panel B, lane 2), a smaller amount of the 94-kDa polypeptide was recovered in the washed membranes and was also phosphorylated. From the soluble fraction, p94 was the predominant polypeptide that was immunoprecipitated and phosphorylated (panel B, lane 1). Other minor components that were phosphorylated were present also in immunoprecipitates obtained with nonimmune rabbit antiserum (see panel A, lane 3). We estimated that the amount of p94 recovered in the membrane fraction constituted less than 1% of the total p94 in HeLa cells, since the membrane fraction usually contained no more than 2% of the total cellular proteins.

To ascertain if p94 kinase is identical to NCP94, we performed peptide map analysis of the phosphorylated proteins that were obtained from the immune complex kinase assays. The 94-kDa phosphoproteins biochemically purified from HeLa cells or immunoprecipitated with anti-peptide antibodies from HeLa and U937 cells yielded peptide maps that were essentially identical (data not shown). These peptide maps were distinct from those generated from p92 immunoprecipitated from U937 cells.

To date, a limited number of cytosolic protein tyrosine kinases have been identified in animal cells. These include products of the fps, FER, and tyk2 genes and a 75-kDa kinase (Wong & Goldberg, 1984; Feldman et al., 1986; Ziocheck et al., 1986; Hao et al., 1989; Firmbach-Kraft et al., 1990). The FER gene product encodes a 94-kDa polypeptide that shows sequence similarity to the fps gene product. In view of the similarity in their apparent molecular weight, we examined the possibility that the HeLa p94 kinase may be the FER gene product. To further ascertain the composition of the isolated enzyme, we fractionated the Mono S pool of the HeLa kinase on a second Mono Q column. A major peak and one minor peak of peptide kinase activity were recovered from the column (Figure 7, panel A). In these column fractions, a 94-kDa polypeptide that coeluted with peptide kinase activity was phosphorylated (panel B). These same fractions also showed immunoreactivity to an antiserum against a TrpE-FER fusion protein (panel C). In fact, the peptide kinase activity, p94 phosphorylation activity, and immunoactivity cofractionated precisely together.

Discussion

We have described identification of a highly active protein tyrosine kinase activity of HeLa cells. Sequential chromatography of the cytosolic fraction resulted in the isolation of a single enzymatic activity. The enzymatic activity cofractionated with a 94-kDa polypeptide that is itself phosphorylated. The majority of p94 was found in the cytosolic fraction of a HeLa lysate, although a small amount of the enzyme was recovered from a membrane fraction. We found that p94 kinase, unlike other PTKs such as receptor kinases, is relatively

resistant to inhibition by genistein. The latter property should be useful in future experiments to investigate biochemical functions of the enzyme.

We used an anti-peptide antiserum to show that the HeLa p94 kinase was likely to be identical to NCP94, which was previously identified in immune complex kinase assays (Fledman et al., 1986). The antiserum was directed against a sequence motif, KQIPVKWTAPEA, that is part of the *v-fps* protein sequence (Shibuya & Hanafusa, 1982). Results of sequence surveys revealed that a consensus motif of PXYWZAPE (where X = I or V, Y = K or R, and Z = T or M) is one of several domains common to most protein tyrosine kinases, but not serine or threonine kinases (Hanks et al., 1988). Since all PTKs whose sequences possess this motif have the ability to autophosphorylate, the ability of the anti-peptide antiserum to immunoprecipitate p94 kinase is consistent with the argument that the 94-kDa polypeptide detected in protein phosphorylation assays was the result of autophosphorylation. Two laboratories have independently identified human and rat cDNA clones that are most likely candidates for sequences that encode human and rat NCP94 (Hao et al., 1989; Letwin et al., 1988). The human cDNA clone, FER, encodes a 94-kDa polypeptide with tyrosine kinase activity (Hao et al., 1989). As Hao et al. noted previously, the FER gene product contains a copy of the sequence KQIPIKWTAPE and should therefore react with the anti-peptide antiserum (Hao et al., 1989). Our results obtained with the anti-peptide antiserum therefore suggest that HeLa p94 kinase may be the FER protein. We further showed that an antiserum to a TrpE-FER fusion protein reacted with HeLa p94 kinase. These data obtained with two antisera with different specificity have provided a basis for concluding that HeLa p94 kinase is likely to be the FER gene product.

During fractionation on the second Mono Q column, a minor additional peak of kinase activity was recovered and was found to cofractionate with a 94-kDa polypeptide also. By V8 peptide map analyses, we have determined that the 94-kDa phosphoprotein recovered in both peaks of activity were identical (unpublished results). The most likely explanation for the resolution of two peaks of kinase activity is that they represent two forms of p94 that have different extents of modification such as phosphorylation.

We showed that p94 kinase phosphorylated a peptide analogue of the cell cycle regulator $p34^{cdc2}$, which is itself a protein kinase [reviewed in Norbury and Nurse (1989)]. It has been shown that in both yeasts and mammals $p34^{cdc2}$ is phosphorylated on serine, threonine, and tyrosine residues and that there is correlation between activation of its kinase activity and dephosphorylation at its tyrosine residue (Gould & Nurse, 1989; Morla et al., 1989). Biochemical and genetic experiments identified Tyr15 as the target site in p34, and that amino acid can be phosphorylated in vitro by $pp60^{v\text{-}src}$ (Draetta et al., 1988; Gould & Nurse, 1989). Two yeast genes, *wee1* and *mik1*, have been identified to be required for tyrosine phosphorylation of Tyr15 of $p34^{cdc2}$ in *Saccharomyces pombe* (Lundgren et al., 1991). However, the corresponding vertebrate enzyme has yet to be discovered. Phosphorylation of the peptide cdcI by HeLa p94 kinase occurred on a single tyrosine residue, which is proximal to a glutamic acid residue. An additional tyrosine in the sequence was phosphorylated only when the predominant phosphorylation site was removed, and then only poorly. These observations showed that the specificity of phosphorylation of Tyr15 of $p34^{cdc2}$ can be duplicated in a small peptide. The results are consistent with the belief that tyrosine residues adjacent to acidic amino acids are preferentially modified by protein tyrosine kinases. Litwin et al. also obtained comparable results using similar peptide analogues (Litwin et al., 1991). However, they reported that a spleen cytoplasmic PTK showed greater activity for the cdc peptide analogue compared to receptor kinases. In contrast, we found that there is no detectable difference in the relative activity of receptor and cytoplasmic kinases using the cdc peptides as substrates (data not shown). Whether or not the differential specificiy is unique to the spleen kinase remains to be further established.

In this report we have provided a biochemical description of the nature and abundance of the predominant tyrosine kinase activity in the cytosol of HeLa cells. We have also demonstrated biochemically that p94 kinase is likely to be identical to NCP94 and the FER gene product, which were previously identified by immunological and cloning methods. These results should facilitate our future efforts to use HeLa cells as a system for studying the biochemistry of p94 kinase function.

ACKNOWLEDGMENTS

We thank Richard Wong and Sean Sullivan for technical assistance and Drs. Tony Pawson and Ricardo Feldman for their generous gifts of reagents. We also thank Dr. Danny Reinberg and Robert Genuario for their comments on the manuscript.

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Structural and Functional Analysis of Human Germ Cell Alkaline Phosphatase by Site-Specific Mutagenesis

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Received July 31, 1991; Revised Manuscript Received January 17, 1992

ABSTRACT: Human germ cell alkaline phosphatase (GCAP), which shares 98% amino acid sequence identity with the placental AP (PLAP), is expressed by malignant trophoblasts. Protein sequence analysis suggests that the Ser residue at position 92 is the putative active site of GCAP which contains two recognition sequences (Asn^{122}-Thr-Thr^{124} and Asn^{249}-Arg-Thr^{251}) for asparagine-linked glycosylation. To examine the roles of the Ser residue and glycan moieties on GCAP activity and processing, we altered the GCAP cDNA by site-directed mutagenesis and expressed the GCAP mutants in COS-1 cells. Substitution of Ser-92 with either a Thr (S92T) or an Ala (S92A) residue yielded a GCAP devoid of catalytic activity, suggesting that the Ser codon 92 is the active site of GCAP. Six GCAP mutants that lack one or both glycosylation sites were constructed by substituting either Asn-122 or Asn-249 with an Asp residue or either Thr-124 or Thr-251 with an Ala residue. The mature GCAP migrated as a 65-kDa product, but GCAP mutants lacking one or both glycosylation sites migrated as 62- or 58-kDa polypeptides, respectively, indicating that both sites were glycosylated. All six glycosylated mutants were active enzymatically and, in addition, were equally sensitive to heat, L-leucine, and EDTA inhibition as the parental enzyme. GCAP as well as its two active-site and six glycosylation mutants could be released from the plasma membrane of transfected COS-1 cells by the proteinase bromelain. This indicates that GCAP is a membrane-bound enzyme located on the outer surface of the plasma membrane and substitution of Ser-92 or removal of oligosaccharide side chains did not prevent membrane anchoring of GCAP. The half-life values of GCAP, S92T, S92A, and the two double-glycosylation mutants were similar (45-46 h). However, the rate of AP synthesis and the total phosphatase activity in cells transfected with a double-glycosylation mutant were reduced when compared with cells transfected with a wild-type or a single-glycosylation mutant. Thus, removing both sugar side chains interferes with enzyme synthesis, but the glycan moieties are not essential for activity, stability, and membrane anchoring of GCAP.

The existence of a distinct placental-like germ cell alkaline phosphatase (GCAP) in humans has been demonstrated by immunological and biochemical analyses (Nakayama et al., 1970; Stigbrand et al., 1982) and recently by molecular cloning (Millan & Manes, 1988; Watanabe et al., 1989). The structures of GCAP (Millan & Manes, 1988; Watanabe et al., 1989) and placental AP (PLAP) (Knoll et al., 1988) genes are very similar. Both are composed of 11 exons and 10 introns and are clustered on the long arm of chromsome 2 (Martin et al., 1987). Mature GCAP and PLAP share 98% amino acid sequence identity and possess similar immunological and physicochemical properties. However, they can be distinguished by differential sensitivities toward heat, EDTA, and the uncompetitive inhibitor L-leucine (Nakayama et al., 1970; Sakiyama et al., 1978). PLAP is primarily expressed in human placenta beginning late in the first trimester of pregnancy (Fishman et al., 1976; Sakiyama et al., 1979). GCAP is found in trace amounts in the testis (Chang et al., 1980) and thymus (Goldstein et al., 1980) and in elevated levels in the serum of patients with germ cell tumors (Wahren et al., 1979; Lange et al., 1982). Recently, we showed that choriocarcinoma cell lines primarily expressed the GCAP (Watanabe et al., 1989), suggesting that malignant transformation of placenta inactivates PLAP but activates GCAP expression. Thus, GCAP provides a valuable marker for studying the molecular mechanisms underlying the transformation process.

GCAP is synthesized as a preproprotein containing both amino- and carboxyl-terminal signal peptides which are cleaved from the nascent protein during processing (Ogata et al., 1988). During enzyme synthesis and processing, the phosphatidylinositol-glycan moiety is covalently attached to the Asp residue 484 of the nascent protein which is then anchored to the plasma membrane. Although the three-dimensional structures of human APs have not been determined, the three-dimensional structure of *Escherichia coli* AP has been deduced from X-ray crystallographic studies (Wyckoff et al., 1983, Kim & Wyckoff, 1991). These studies indicate that the amino acid residues that constitute the active-site pocket of *Escherichia coli* AP include an Arg residue, the ligands to the three metal binding sites, and a Ser residue that binds phosphate. Sequence alignment and conservation of mammalian

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