Segel, I. H. (1975) *Enzyme Kinetics*, pp 591-593. John Wiley & Sons, New York.
Shibuya, M., & Hanafusa, H. (1982) *Cell 30*, 787-795.
Sullivan, S., & Wong, T. W. (1991) *Anal. Biochem. 197*, 65-68.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203-212.
Veillette, A., Bookman, M. A., Horak, E. M., & Bolen, J. B. (1988) *Cell 55*, 301-308.
Wong, T. W., & Goldberg, A. R. (1984) *J. Biol. Chem. 259*, 8505-8512.
Zioncheck, T. F., Harrison, M. L., & Geahlen, R. L. (1986) *J. Biol. Chem. 261*, 15637-15643.

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

Takeshi Watanabe, Naohiro Wada, and Janice Yang Chou*
Human Genetics Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

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

* To whom correspondence should be addressed at Building 10, Room 9S242, NIH, Bethesda, MD 20892.

and *Escherichia coli* APs suggest that the Ser residue at position 92 would be the putative active site of GCAP (Millan, 1988). This Ser codon is located between the Asp and Gly residues, and the Asp-Ser-Gly sequence corresponds to the characteristic sequence Asp/Glu-Ser-Ala/Gly found in most serine proteases and eukaryotic APs (Millan, 1988). In this study, we performed oligonucleotide-directed mutagenesis to substitute Ser-92 with either a Thr or an Ala residue and demonstrated that the active-site pocket of GCAP includes this Ser residue.

Human APs exist as dimers of identical glycopolypeptides (Sussman, 1984). In GCAP and PLAP, the positions of the two asparagine (N)-linked glycosylation sites are conserved, suggesting that these sugar side chains may have a common functional role (Millan, 1986; Knoll et al., 1987; Watanabe et al., 1989). The glycan moieties of glycoproteins have been shown to play important roles including maintenance of polypeptide conformation and solubility, protection of the polypeptide chain from proteolytic degradation, and production of signals for intracellular sorting and externalization [for review, see Olden et al. (1982, 1985)]. More recently, the sugar side chains were found to be essential for the catalytic activity of human lipoprotein lipase (Semenkovich et al., 1990) and the assembly and secretion of human chorionic gonadotropin (hCG; Matzuk et al., 1989). Using tunicamycin, an inhibitor of protein glycosylation (Struck & Lennarz, 1980), to inhibit the addition of N-linked carbohydrates to GCAP in choriocarcinoma cells, we showed that the degradation rates of glycosylated and nonglycosylated forms of this phosphatase were similar (Ito & Chou, 1984b). Thus, the stability of GCAP appears unaltered after eliminating its N-linked oligosaccharide side chains. However, tunicamycin removes indiscriminately all oligosaccharide units, and thus cannot be used to analyze individual glycosylation sites in multiglycosylated proteins. In the present report, we examined the extent of protein glycosylation in GCAP and the role of the oligosaccharide units on GCAP enzyme activity, protein stability, and membrane anchoring by eliminating one or both of the consensus glycosylation sequences by oligonucleotide-directed mutagenesis. Our data show that GCAP mutants lacking N-linked oligosaccharide side chains are enzymatically active and anchored to the plasma membrane. Removing both glycan moieties does not alter the half-life of GCAP. However, GCAP mutants lacking both glycan moieties are synthesized at a reduced rate resulting in a decrease in total phosphatase activity.

Materials and Methods

Oligonucleotide-Directed Mutagenesis. Mutagenesis was carried out as described by Kunkel et al. (1987) using an oligonucleotide-directed in vitro mutagenesis kit obtained from Bio-Rad Laboratories (Richmond, CA). GCAP cDNA used for mutagenesis contains nucleotides 35–2144 of GCAP (Watanabe et al., 1989), which consists of an 18 bp 5′-untranslated sequence, the entire coding region (nucleotides 53–1648) and a 496 bp 3′-untranslated sequence. pSVLGCAP is GCAP cDNA subcloned into the pSVL vector (Pharmacia, Piscataway, NJ). pSVLGCAP-5′ was generated by removing the *Bam*HI fragment (nucleotides 268–2144) of GCAP from pSVLGCAP. Oligonucleotides synthesized on a Cyclone Plus DNA synthesizer (Milligen Biosearch, Navato, CA) were 5′-phosphorylated and annealed to a single-stranded M13mp19 template containing the *Bam*HI fragment (nucleotides 268–2144) of GCAP cDNA. After mutagenesis, the *Bam*HI fragment containing the mutated sequence was excised from M13mp19 and recloned into pSVLGCAP-5′ which contains nucleotides 35–267 of GCAP. Mutants were confirmed by hybridization with the oligonucleotide probe and by direct sequencing of mutants in the pSVL vector. For each mutant, the approximately 300 bp sequence around the mutation site in both strands was determined.

Expression in COS-1 Cells. COS-1 cells were grown at 37 °C in Hepes-buffered Dulbecco's modified minimal essential medium supplemented with streptomycin, penicillin, and 10% fetal bovine serum. pSVLGCAP, pSVLPLAP [containing nucleotides 1 to the 3′ end of PLAP cDNA (Watanabe et al., 1989) in the pSVL vector], or pSVLGCAP mutant generated by site-specific mutagenesis was transfected into COS-1 cells (10 μg/25 cm^2 flask of cells) by the DEAE-dextran/chloroquine method essentially as described (Okayama et al., 1987). After a 72-h incubation, cells were lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, and 0.25% deoxycholate and heated at 55 °C for 20 min to inactivate endogenous heat-labile AP. The heat-inactivated extracts were used for measuring enzyme activity and protein content.

To examine AP biosynthesis, transfected COS-1 cells were labeled for 3 h with L-[^{35}S]methionine (100 μCi/mL; ICN Biochemicals, Inc., Lisle, IL) in the absence or presence of tunicamycin (1 μg/mL) or labeled for 4 h with [^{3}H]glucosamine (100 μCi/mL; Du Pont–New England Nuclear). After cells were labeled, they were washed twice with phosphate-buffered saline (PBS) and treated with 1 mL of lysis solution containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 100 μg of trypsin inhibitor in PBS. Polypeptides in cell extracts and medium samples were isolated by immunoprecipitation with rabbit antiserum to PLAP (Ito & Chou, 1984a) and analyzed by 10% polyacrylamide–SDS gel electrophoresis and fluorography. Apparent molecular weights were determined using [^{14}C]methionine-labeled protein standards (Amersham Corp., Arlington Height, IL).

Alkaline Phosphatase Assay. AP activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 10.7 and 37 °C (Sakiyama et al., 1978). By definition, 1 unit of enzyme releases 1 μmol of *p*-nitrophenol per minute. Enzyme activities between treatments were compared using a Student's "*t*"-test.

Nucleic Acid Hybridization Analysis. Total RNA was extracted by the guanidinium thiocyanate method of Chirgwin et al. (1979), separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (Lehrach et al., 1977), and transferred to Zetabind membranes (AMF, Meriden, CT). The filters were hybridized with a nick-translated, ^{32}P-labeled, 568 bp *Sst*I–*Sst*I insert of a genomic clone containing nucleotides 2233–2800 of the GCAP gene (Watanabe et al., 1989) or with a probe containing the 3′ *Eco*RI–*Bam*HI fragment of the human β-actin gene (Ponte et al., 1983). Hybridization in the presence of dextran sulfate and washing were performed as previously described (Watanabe et al., 1989).

Bromelain Treatment. Release of GCAP from transfected COS-1 cells by bromelain was performed essentially as described (Berger et al., 1988). Briefly, the transfected COS-1 cultures were washed twice with PBS and twice with a releasing buffer containing 25 mM Tris-HCl, pH 7.5, 0.25 M sucrose, and 10 mM glucose and then incubated in releasing buffer (1 mL/25 cm^2 flask) containing bromelain (2 units/mL) for 2 h at 37 °C. Cell suspensions were centrifuged at 10000*g* for 2 min, and AP activities in cell pellets and supernatant solutions were determined.

To analyze AP synthesis in bromelain-treated cultures, the transfected COS-1 cells were labeled with L-[^{35}S]methionine

A. Wild-Type and Mutant GCAP

WT	Asn^{122}-Thr-Thr^{124} ---- Asn^{249}-Arg-Thr^{251}
ΔAsn 1	**Asp^{122}**-Thr-Thr^{124} ---- Asn^{249}-Arg-Thr^{251}
ΔThr 1	Asn^{122}-Thr-**Ala^{124}** ---- Asn^{249}-Arg-Thr^{251}
ΔAsn 2	Asn^{122}-Thr-Thr^{124} ---- **Asp^{249}**-Arg-Thr^{251}
ΔThr 2	Asn^{122}-Thr-Thr^{124} ---- Asn^{249}-Arg-**Ala^{251}**
ΔAsn (1+2)	**Asp^{122}**-Thr-Thr^{124} ---- **Asp^{249}**-Arg-Thr^{251}
ΔThr (1+2)	Asn^{122}-Thr-**Ala^{124}** ---- Asn^{249}-Arg-**Ala^{251}**

B. Oligonucleotides for Site-Specific Mutagenesis

WT (464-488)	5'-TGCCGCGTGTCGTGTTGCACTGGTT-3'
ΔAsn 1	5'-GTGTCGTGTCGCACTGGTT-3'
ΔThr 1	5'-TGCCGCGTGCCGTGTTGCA-3'
WT (845-869)	5'-GGAGCTCAGTGCGGTTCCACACGTA-3'
ΔAsn 2	5'-CAGTGCGGTCCCACACGTA-3'
ΔThr 2	5'-GGAGCTCAGCGCGGTTCCA-3'
WT (Ser^{92}) (374-393)	5'-GTGGCTCCACTGTCTGGCAC-3'
S92T (Thr^{92})	5'-GTGGCTCCAGTGTCTGGCAC-3'
S92A (Ala^{92})	5'-GTGGCTCCAGCGTCTGGCAC-3'

FIGURE 1: Amino acid substitutions in the six glycosylation mutants of GCAP (A) and oligonucleotides used for constructing GCAP mutants by site-specific mutagenesis (B).

(100 μCi/mL) for 3 h. After the cells were labeled, cultures were treated with bromelain for 2 h at 37 °C, and cell pellets were separated from the supernatants by centrifugation. AP polypeptides in cell pellets and supernatant solutions were isolated by immunoprecipitation and analyzed by gel electrophoresis and fluorography.

Results

Serine Residue at Position 92 Is the Active Site of GCAP. Although the active sites of mammalian APs have not been demonstrated directly, a Ser residue in PLAP has been shown to bind inorganic phosphate covalently (Whitaker et al., 1976). Furthermore, sequence alignment and conservation between *Escherichia coli* and mammalian APs suggest that the Ser residue at position 92 in the sequence Asp-Ser-Gly is the putative active site of GCAP (Millan, 1988). Accordingly, we employed site-specific mutagenesis to replace Ser-92 with either a Thr (S92T) or an Ala (S92A) residue (Figure 1) and examined AP activity and biosynthesis after transfecting the wild-type and mutant GCAPs into COS-1 cells. No AP activity or synthesis was detected in any culture media. In cell extracts, significant amounts of phosphatase activity were found in COS-1 cells transfected with the wild-type GCAP (Table I). Substitution of the Ser at codon 92 with either a Thr (S92T) or an Ala (S92A) resulted in the complete abrogation of GCAP enzyme activity (Table I). The lack of AP catalytic activity of the two active-site mutants was not due to a lack of enzyme synthesis. Mutant phosphatases comigrating with the wild-type GCAP were actively synthesized by transfected cells (Figure 2).

To demonstrate that GCAP and its two active-site mutants, S92T and S92A, have similar stability, we examined the rates

Table I: AP Enzyme Activity in Transfected COS-1 Cells[a]

AP	AP act. (milliunits/mg of cellular protein)	AP sp act. (milliunits/10^{-3} cpm)	sensitivity (% inhibtn)	
			L-leucine	EDTA
JEG-GCAP	170.4 ± 9.2		77.0 ± 5	76.0 ± 7
GCAP	98.6 ± 10.4	15.2	91.8 ± 1.5	78.0 ± 6.7
PLAP	82.5 ± 0.8		30.0 ± 1.4	11.1 ± 5
S92T	ND			
S92A	ND			
ΔAsn 1	137.0 ± 14	11.7	89.6 ± 0.8	72.8 ± 2.5
ΔThr 1	110.0 ± 7	12.3	82.7 ± 0.4	76.1 ± 0.2
ΔAsn 2	79.3 ± 10	15.0	83.2 ± 2.8	77.6 ± 5.4
ΔThr 2	104.5 ± 18.5	12.9	85.7 ± 0.5	78.6 ± 2.1
ΔAsn (1+2)	36.3 ± 2	12.7	76.6 ± 1.9	63.6 ± 9.3
ΔThr (1+2)	24.8 ± 2	12.2	79.6 ± 5.6	73.4 ± 8.9

[a] AP activities in COS-1 cells transfected with GCAP, PLAP, or GCAP glycosylation mutants were determined in the absence or presence of L-leucine (10 mM) or EDTA (10 mM). To inactivate endogenous heat-labile AP in COS-1 cells, cell extracts were heated for 20 min at 55 °C prior to phosphatase assay. AP activities in mock-infected cells, before and after heat treatment, were 1.3 and 0.8 milliunits/mg of cellular protein, respectively. JEG-GCAP was AP activity of JEG-3 choriocarcinoma cells which express the GCAP gene (Watanabe et al., 1989). Data are presented as the mean ± SE. ND represents nondetectable. AP activity was the enzyme activity in transfected COS-1 cells per milligram of cellular protein, and AP specific activity was the ratio of enzyme activity in transfected COS-1 cells to the [^{35}S]methionine radioactivity incorporated into newly synthesized AP protein.

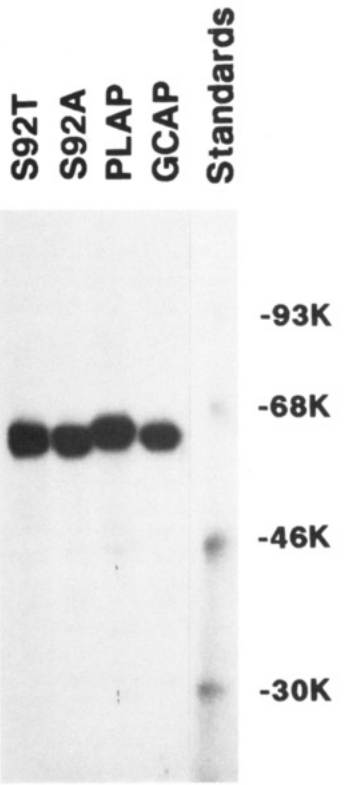

FIGURE 2: In vitro synthesis of wild-type GCAP, PLAP, and GCAP active-site mutants. COS-1 cells transfected with the wild-type GCAP (Ser^{92}), PLAP, GCAP S92T (Thr^{92}), or S92A (Ala^{92}) mutant were pulse-labeled with L-[^{35}S]methionine for 3 h. The newly synthesized APs were isolated by immunoprecipitation and analyzed by polyacrylamide–SDS electrophoresis and fluorography as described under Materials and Methods.

of protein degradation of wild-type and mutant GCAPs by pulse–chase experiments. COS-1 cells transfected with the wild-type GCAP or the S92T or S92A mutant were pulsed-labeled with L-[^{35}S]methionine for 90 min and then subjected to chase with fresh medium containing 500 μg/mL L-methionine for 6, 12, 24, 36, 48, 60, and 72 h. APs in cell lysates were isolated by immunoprecipitation, and the radio-

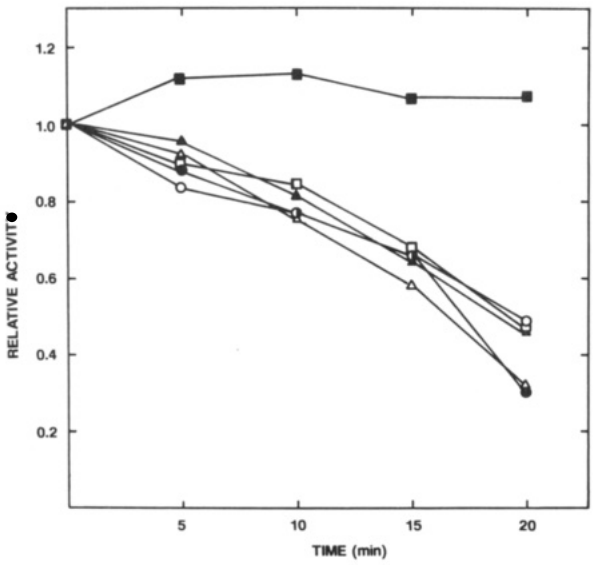

FIGURE 3: Heat stabilities of PLAP, wild-type GCAP, and GCAP glycosylation mutants. APs in transfected COS-1 cells were heated at 55 °C in 0.3 M 2-amino-2-methyl-1-propanol, pH 10, at the time intervals indicated. Relative activity is the proportion of phosphatase remaining in comparison with the activity at zero time which is 1.0. (■) PLAP; (□) GCAP; (●) ΔAsn 1; (○) ΔAsn 2; (▲) ΔAsn (1+2); (△) ΔThr (1+2).

activity incorporated into the GCAP was estimated by liquid scintillation counting. The half-life values of all three Aps were approximately 45–46 h (data not shown). Therefore, substitution of Ser at position 92 did not grossly alter the conformation of GCAP.

Characterization of GCAP Glycosylation Mutants Generated by Site-Specific Mutagenesis. GCAP contains two recognition sequences, Asn^{122}-Thr-Thr^{124} and Asn^{249}-Arg-Thr^{251}, for N-linked glycosylation (Millan & Manes, 1988; Watanabe et al., 1989). To assess the functional importance of the N-linked oligosaccharide units, we constructed six mutants that eliminated one (ΔAsn 1, ΔThr 1, ΔAsn 2, or ΔThr 2) or both [ΔAsn (1+2) or ΔThr (1+2)] glycosylation sites (Figure 1). These mutants were obtained by mutating one or both the Asn residues at positions 122 and 249 to Asp or one or both of the Thr residues at positions 124 and 251 to Ala. After mutagenesis, the coding region of wild-type or mutant GCAP cDNA was subcloned into the pSVL vector and used for transfection studies. COS-1 cells transfected with the wild-type GCAP expressed enzymatically-active AP which was inhibited by L-leucine and EDTA to the same degree as the GCAP synthesized by JEG-3 choriocarcinoma cells (Table I). Moreover, the in vitro expressed GCAP, like the choriocarcinoma enzyme, was more sensitive to heat inactivation than the PLAP (Figure 3). The in vitro expressed PLAP was also more resistant than the GCAP to L-leucine and EDTA inhibition (Table I) which are characteristics of the PLAP (Nakayama et al., 1970; Sakiyama et al., 1978).

The six GCAP glycosylation mutants were enzymatically active. Moreover, all mutants were similarly inhibited by L-leucine, EDTA (Table I), and heat (Figure 3) as the wild-type GCAP. However, total AP activity, not the specific enzyme activity, was considerably lower in cells transfected with a double-glycosylation mutant as compared with cells transfected either with the wild-type or with a single-glycosylation mutant (Table I).

To examine AP synthesis, COS-1 cells were labeled with [^{35}S]methionine in the absence or presence of tunicamycin and the newly synthesized AP polypeptides analyzed by SDS–polyacrylamide gel electrophoresis (Figure 4A). In the absence of tunicamycin, GCAP existed as two glycopolypeptides of 65 and 63 kDa, which were previously shown to differ in the amounts of neuraminic acid residues of the sugar side chains (Ito & Chou, 1984b). After limited tunicamycin treatment, polypeptides of 62 and 58 kDa were produced which may represent the singly-glycosylated and nonglycosylated forms of GCAP monomer (Figure 4A). GCAP lacking either of the two glycosylation sites migrated as a 62-kDa polypeptide in the absence of tunicamycin and as a 58-kDa product in its presence (Figure 4A). Mutants lacking both glycosylation sites

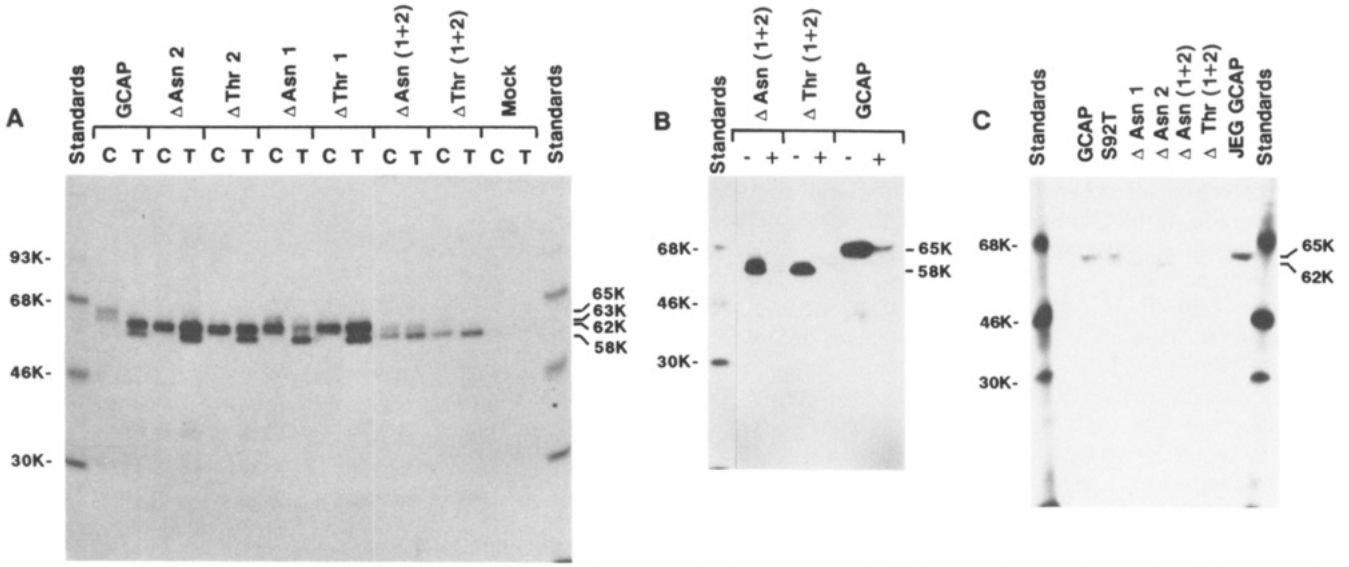

FIGURE 4: Analysis of in vitro expressed wild-type GCAP and its glycosylation mutants. (A) Biosynthesis. Mock-transfected COS-1 cells or cells transfected with wild-type GCAP or GCAP mutant that lacks one (ΔAsn 1, ΔThr 1, ΔAsn 2, or ΔThr 2) or both [ΔAsn (1+2) or ΔThr (1+2)] glycosylation sites were pulsed-labeled with L-[^{35}S]methionine for 3 h in the absence (C) or presence of tunicamycin (T, 1 μg/mL). (B) Competition for immunoprecipitation. COS-1 cells transfected with wild-type GCAP or a GCAP mutant that lacks both [ΔAsn (1+2) or ΔThr (1+2)] glycosylation sites were labeled with L-[^{35}S]methionine for 3 h. (C) Incorporation of [^{3}H]glucosamine. COS-1 cells transfected with the wild-type GCAP, GCAP S92T mutant, or GCAP mutant that lacks one (ΔAsn 1 or ΔAsn 2) or both [ΔAsn (1+2) or ΔThr (1+2)] glycosylation sites were pulsed-labeled with [^{3}H] glucosamine for 4 h. APs in cell lysates were isolated by immunoprecipitation with anti-PLAP serum in the presence of 0.5 μg [pulse or competition (−)] or 25 μg [competition (+)] of carrier PLAP and analyzed by polyacrylamide–SDS gel electrophoresis and fluorography.

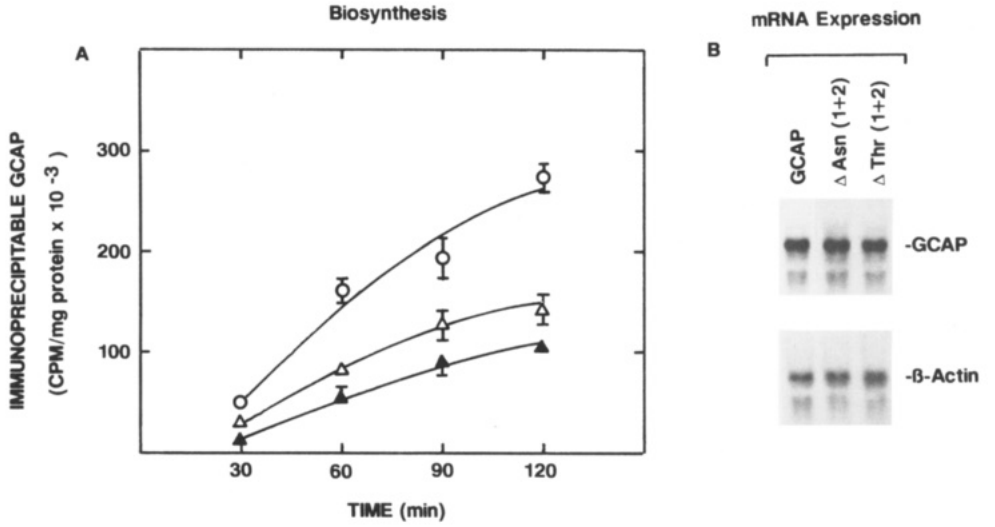

FIGURE 5: Biosynthesis and mRNA expression of wild-type GCAP and its double-glycosylation mutants. COS-1 cells transfected with the wild-type GCAP or a mutant GCAP that lacks both glycosylation sites [ΔAsn (1+2) or ΔThr (1+2)] were incubated for 3 days. (A) Biosynthesis. On day 3, cells were pulsed-labeled with L-[^{35}S]methionine for 30, 60, 90, and 120 min. APs in cell lysates were isolated by immunoprecipitation, and radioactivity incorporated into the GCAP was estimated by liquid scintillation counting. Data points are derived from triplicate cultures, each analyzed in duplicate. Each point represents the mean ± SEM. (○) Wild-type GCAP; (▲) ΔAsn (1+2); (△), ΔThr (1+2). (B) mRNA expression. On day 3, cells were lysed for RNA isolation. Total RNA (10 μg/lane) was separated by agarose gel electrophoresis, transferred to a Zetabind membrane, and hybridized with a GCAP probe as described under Materials and Methods. After removal of bound GCAP by washing the filter in 95% formamide (60 °C for 30 min), the filter was rehybridized with a human β-actin probe.

were unaffected by tunicamycin, and a 58-kDa polypeptide was the major product synthesized both in the absence and in the presence of this antibiotic. We do not know why a tunicamycin-resistant polypeptide of 61 kDa was routinely observed in the double mutants whose Asn residues at positions 122 and 249 were mutated to Asp. In agreement with studies on AP activity, GCAP synthesis in cells transfected with double-glycosylation mutants was lower than in cells transfected with the wild-type or a single-glycosylation mutant (Figure 4A). Thus, biosynthesis of GCAP that lacks both glycan moities was reduced, resulting in a decrease in total phosphatase activity.

The specificities of the in vitro synthesized GCAP polypeptides were confirmed by competition experiments (Figure 4B). Immunoprecipitation of 65- and 58-kDa polypeptides as well as the 61-kDa polypeptide was prevented by excess PLAP which shares immunological determinants with GCAP (Ito & Chou, 1984b).

The ability of GCAP glycosylation mutants to incorporate [^{3}H]glucosamine, a precursor of the oligosaccharide units, was then examined (Figure 4C). Both wild-type GCAP and the single-glycosylation mutants incorporated glucosamine, although the wild-type enzyme incorporated approximately double the amounts of this precursor. As expected, mutants that lack both consensus sequences for N-linked glycosylation failed to incorporate glucosamine.

GCAP Double-Glycosylation Mutants Are Synthesized at Reduced Rates. The decrease in the rate of accumulation of GCAP that lacks both glycan moieties may be the consequence of a reduced rate of protein synthesis or a change in GCAP stability. Using tunicamycin to inhibit protein glycosylation in choriocarcinoma cells, we showed that the degradation rates of glycosylated and nonglycosylated GCAPs were similar, suggesting that the stability of this phosphatase was unaltered after elimination of the glycan moieties (Ito & Chou, 1984b). To demonstrate that the in vitro synthesized double-glycosylation mutants of GCAP have similar stability as the parental enzyme, we examined the rates of protein degradation of wild-type and mutant GCAPs by pulse–chase experiments as described for the active-site mutants. The half-life values of the ΔAsn (1+2) and the ΔThr (1+2) mutants were indistinguishable from that of the glycosylated wild-type GCAP (45–46 h, data not shown). Therefore, the glycan moieties do not play a role in protecting the enzyme against proteolytic degradation.

To examine whether the observed reduction in AP protein accumulation and total enzyme activity in cells transfected with a GCAP double-glycosylation mutant may result from a decrease in the rate of AP biosynthesis, we examined the rates of synthesis of wild-type and mutant GCAP (Figure 5A). The rate of synthesis of either the ΔAsn (1+2) or the ΔThr (1+2) mutant was lower than that of the wild-type enzyme (Figure 5A). Two hours after pulse-labeling, the amount of immunoprecipitable GCAP in cells transfected with the ΔAsn (1+2) or the ΔThr (1+2) mutant was approximately 37% or 52%, respectively, of the level in cells transfected with the wild-type GCAP.

COS-1 cells transfected with the wild-type GCAP or its double-glycosylation mutants contained similar levels of GCAP as well as β-actin mRNA (Figure 5B). Thus, the decrease in synthesis of mutant lacking both glycan moieties was not due to a reduction in GCAP mRNA expression.

GCAP Glycosylation Mutants Are Present on the Outer Surface of the Plasma Membrane. It has been shown that mature GCAP or PLAP is attched to the plasma membrane of cultured cells via a COOH-terminal phosphatidylinositol-glycan moiety (Ogata et al., 1988; Low & Saltiel, 1988; Micanovic et al., 1988). PLAP is located on the outer surface of the plasma membrane and can be released from cells by phosphatidylinositol-specific phospholipase C or bromelain. The latter is a protease that is known to remove a 2-kDa segment from PLAP near its COOH terminus (Jemmerson et al., 1984). The strong sequence similarity between these two phosphatases suggests that GCAP may also be present on the outer surface of the plasma membrane. We therefore examined membrane localization of GCAP in transfected COS-1 cells by treatment with bromelain. Cells expressing wild-type GCAP were labeled with [^{35}S]methionine and treated with bromelain, and the newly synthesized GCAPs in cell lysates or supernatants solutions were analyzed by SDS–

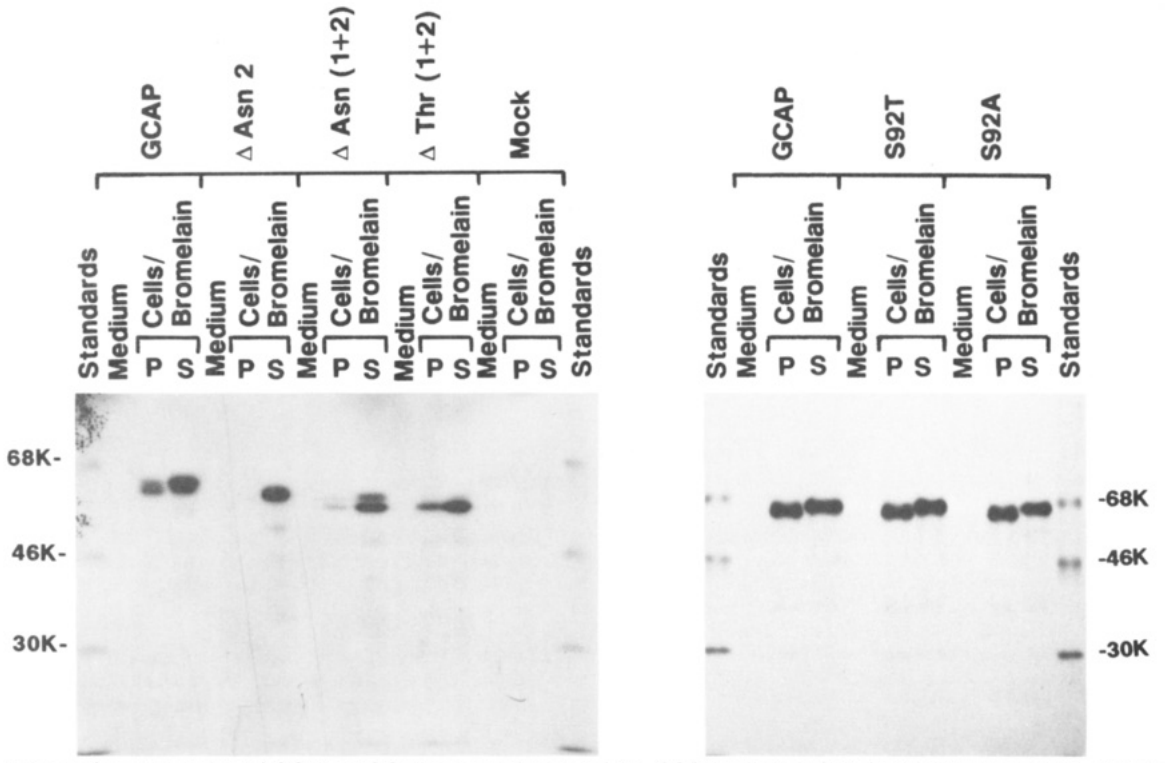

FIGURE 6: Release of membrane-bound GCAP or GCAP mutants by bromelain. COS-1 cells transfected with wild-type GCAP, GCAP mutants that lack one (ΔAsn 2) or both [ΔAsn (1+2) or ΔThr (1+2)] glycosylation sites, or GCAP active-site mutants (S92T or S92A) were pulsed-labeled with L-[^{35}S]methionine for 3 h. AP in medium samples was isolated by immunoprecipitation and analyzed by polyacrylamide–SDS gel electrophoresis and fluorography. For bromelain treatment, the labeled cells were incubated for 2 h at 37 °C in a buffer containing bromelain as described under Materials and Methods. APs remaining in cell lysates (P) or released into the supernatant solutions (S) were isolated by immunoprecipitation and analyzed by polyacrylamide–SDS gel electrophoresis and fluorography.

polyacrylamide gel electrophoresis (Figure 6). Prior to bromelain treatment, wild-type GCAP was exclusively cell-associated as no GCAP polypeptide was detected in the culture medium. After 2-h treatment with bromelain, over 50% of wild-type GCAP protein was released into the supernatant solution, indicating that GCAP, like PLAP, was located on the outer surface of the plasma membrane.

To determine whether GCAP active-site mutants or mutants lacking N-linked oligosaccharide chains are processed normally and inserted into the plasma membrane, the sensitivities of these GCAP mutants to release by bromelain were examined (Figure 6). The newly synthesized mutant polypeptides were largely released into the supernatant solutions by bromelain. This demonstrates that substitution of Ser-92 with either Thr (S92T) or Ala (S92A) or GCAP lacking one or both glycosylation sites was membrane-associated and located on the outer surface of the plasma membrane.

To ascertain that GCAP and its glycosylation mutants released by bromelain represent the active cell-surface enzymes, phosphatase activities in cell lysates and supernatant solutions were determined (Table II). In agreement with labeling experiments, the majority of GCAP activities (58–69%) were released into the supernatant solutions after bromelain treatment. Moreover, we detected no apparent variation in sensitivity to bromelain between wild-type and glycosylation mutants. Thus, the N-linked glycan moieties are not essential for membrane insertion of GCAP.

Table II: Release of Membrane-Bound AP by Bromelain[a]

AP mutant	transfected COS-1 cell act. (milliunits/mg of cellular protein)	
	pellet	supernatant
GCAP	86.5 ± 5.5	149.2 ± 11.8 (63.0)
ΔAsn 1	68.0 ± 2.7	119.2 ± 9.1 (63.6)
ΔAsn 2	81.0 ± 4.5	146.5 ± 1.8 (64.4)
ΔAsn (1+2)	41.3 ± 3.6	90.1 ± 8.2 (68.8)
ΔThr (1+2)	54.6 ± 5.5	74.6 ± 4.5 (57.7)

[a] COS-1 cells transfected with GCAP or its glycosylation mutants were treated with bromelain for 2 h at 37 °C. AP activities in cell pellets and supernatant solutions were determined as described under Materials and Methods. Numbers in parentheses represent percent of activity released into the supernatant solutions.

Discussion

In the present study, we examined the structure–function relationship of GCAP using site-directed mutagenesis to alter the primary sequence and analyze the altered proteins in COS-1 cells. Our results showed that substitution of either a Thr (S92T) or an Ala (S92A) for the Ser residue at position 92 yielded a GCAP devoid of phosphatase activity. However, S92T and S92A mutants, like the wild-type GCAP, yielded approximately the same amount of immunoprecipitable GCAP proteins, had similar stabilities, and anchored to the outer surface of the plasma membrane. Thus, the Ser-92 in the context of Asp-Ser-Gly is absolutely essential for the catalytic activity of GCAP. The active site of *Escherichia coli* AP and the putative active sites of all mammalian APs isolated to date contain the characteristic sequence Asp-Ser-Gly/Ala which is also found in most serine proteases (Millan, 1988). Studies have suggested that this Asp residue may play a role in the stabilization of the active site (Wyckoff et al., 1983; Millan, 1988).

X-ray crystallographic studies showed that the active center of *Escherichia coli* AP includes, in addition to the reactive Ser, an Arg residue and the ligands to the three metal binding sites (Wyckoff et al., 1983; Kim & Wyckoff, 1991). The accessibility calculations based on the refined coordinates of the *Escherichia coli* enzyme (Sowadski et al., 1985) show that the active-site pocket barely accomodates an inorganic phosphate.

The inactivation of GCAP by the Thr for Ser-92 substitution suggests that the structure of the GCAP active center is also rather rigid. A Thr residue at position 92 either destroyed the enzyme's active center structure or rendered the center inaccessible to substrate or phosphate. Therefore, the tertiary structure of the AP active center is very complex. It should be possible to elucidate the structure–function relationships for each of these sites using site-specific mutagenesis and the in vitro expression system described.

GCAP, as well as PLAP, contains two potential N-linked glycosylation sites (Millan, 1986; Millan & Manes, 1988; Watanabe et al., 1989). However, Millan (1986, 1988) stated that only the Asn-249 is glycosylated in the mature PLAP. Evidence presented in this study demonstrates that both N-linked glycosylation sites are utilized in GCAP. The in vitro expressed wild-type GCAP is a 65-kDa polypeptide which comigrated with the fully processed GCAP monomer synthesized by human choriocarcinoma cells. GCAP mutants that lack one or both glycosylation sites migrated as polypeptides of 62 or 58 kDa, respectively. Thus, the apparent molecular masses of the non-, singly-, and doubly-glycosylated GCAP monomers are 58, 62, and 65 kDa, respectively. Although we do not have direct evidence to show that both potential sites in PLAP are glycosylated, the similarities in apparent molecular masses of glycosylated and nonglycosylated GCAP and PLAP (Watanabe et al., 1989) suggest that both sites may be utilized in the PLAP.

The N-linked oligosaccharide chains have been shown to play a variety of functional roles (Olden et al., 1982, 1985). In earlier studies (Ito & Chou, 1984b), we showed that the stabilities of glycosylated and nonglycosylated GCAPs synthesized by choriocarcinoma cells in the absence or presence of tunicamycin were similar. We now show that the half-time values of wild-type GCAP and its two double-glycosylation mutants are similar. Therefore, the sugar glycans are not required for protecting the polypeptides chain from proteolytic degradation. In addition, we demonstrate that the glycan moieties are not essential for GCAP enzyme activity and play no essential role in membrane anchoring of AP. First, the catalytic activities of the fully processed and singly- and non-glycosylated GCAPs were similar. Moreover, wild-type GCAP and its six glycosylation mutants exhibited similar sensitivities toward L-leucine, EDTA, and heat inhibition. Second, both phosphatase protein and activity could be released from COS-1 cells by bromelain treatment, regardless of whether these cells were transfected with the wild type or a mutant GCAP lacking one or both glycosylation sites. Bromelain has been shown to cleave PLAP near its COOH terminus (Jemmerson et al., 1984) and release this phosphatase from the plasma membrane (Low & Saltiel, 1988; Micanovic et al., 1988). Furthermore, GCAP is tailed with a phosphatidylinositol-glycan moiety before membrane insertion (Ogata et al., 1988). Thus, GCAP and also its glycosylation mutants are membrane-associated proteins that are phosphatidylinositol-glycan-tailed and anchored to the outer surface of the plasma membrane. Removing the N-linked oligosaccharides at positions 122 and 249 had no adverse effects on GCAP tailing, membrane anchoring, and externalization.

Earlier studies (Ito & Chou, 1984b) have shown that the rate of GCAP synthesis was reduced by tunicamycin, an inhibitor of N-linked protein glycosylation (Struck & Lennarz, 1980). Since the translatable GCAP mRNA was decreased in the presence of tunicamycin, it was not possible to determine whether removing the glycan moieties affected GCAP biosynthesis. In the present study, we show that the rates of synthesis of the GCAP double-glycosylation mutants were lower than those for the wild-type enzyme. As a result, the level of total phosphatase activity in COS-1 cells transfected with a mutant that lacks both N-linked oligosaccharide side chains was lower than in cells transfected with the wild-type GCAP. The importance of glycan moieties in protein biosynthesis is not unique to GCAP. The N-linked glycosylation has been shown to be essential for the level of biosynthesis of the glycoprotein hormone erythropoietin (Dube et al., 1988). Similarly, elimination of the N-linked oligosaccharide chains inhibits synthesis of the vesicular stomatitis virus G protein, while the degradation rates of the nonglycosylated and glycosylated G proteins were similar (Machamer et al., 1985). However, these studies did not carry out detailed kinetic analyses of synthesis and degradation. At the present time, the molecular mechanisms responsible for the decrease in the synthetic rates of nonglycosylated glycoproteins are not well understood. It has been well established, however, that glycosylation occurs contranslationally; for example, peptide chain elongation, glycosylation, and folding take place concurrently (Kornfeld & Kornfeld, 1985). Other studies have indicated that the glycan moiety is important in the maintenance of the correct polypeptide conformation (Olden et al., 1982, 1985). Moreover, oligosaccharide units have been shown to play an essential role in the dimerization of the hCG α- and β-subunits (Matzuk & Boime, 1988; Matzuk et al., 1989). Approximately 40% of the secreted deglycosylated hCG-α fails to combine with the native or deglycosylated hCG-β (Matzuk & Boime, 1988). GCAP is a membrane-associated glycoprotein whose active form is a homodimer (Sussman, 1984). Thus, it is possible that double-glycosylation mutants, although phosphatidylinositol-glycan-tailed and eventually anchored to the plasma membrane, were dimerized and/or transported more slowly than either the wild-type or the single-glycosylation mutants. The delayed dimerization and/or transportation in turn may decrease the rate of GCAP synthesis and total enzyme activity in transfected COS-1 cells.

ACKNOWLEDGMENTS

We thank Drs. C. A. Plouzek, J. K. Ritter, and L. Shelly for critical reading of the manuscript.

Registry No. AP, 9001-78-9; Ser, 56-45-1.

REFERENCES

Berger, J., Howard, A. D., Brink, L., Gerber, L., Hauber, J., Cullen, B. R., & Udenfriend, S. (1988) *J. Biol. Chem. 263*, 10016–10021.

Chang, C. H., Angellis, D., & Fishman, W. H. (1980) *Cancer Res. 40*, 1506–1510.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Dube, S., Fisher, J. W., & Powell, J. S. (1988) *J. Biol. Chem. 263*, 17516–17521.

Fishman, L., Miyayama, H., Driscoll, S. G., & Fishman, W. H. (1976) *Cancer Res. 36*, 2268–2273.

Goldstein, D. J., Rogers, C., & Harris, H. (1980) *Clin. Chim. Acta 125*, 63–75.

Ito, F., & Chou, J. Y. (1984a) *J. Biol. Chem. 259*, 2526–2530.

Ito, F., & Chou, J. Y. (1984b) *J. Biol. Chem. 259*, 14997–14999.

Jemmerson, R., Shah, N., Takaya, M., & Fishman, W. H. (1984) *Prog. Clin. Biol. Res. 166*, 105–115.

Kim, E. E., & Wyckoff, H. C. (1991) *J. Mol. Biol. 218*, 449–464.

Knoll, B. J., Rothblum, K. N., & Longley, M. (1987) *Gene 60*, 267–276.

Knoll, B. J., Rothblum, K. N., & Longley, M. (1988) *J. Biol. Chem. 263*, 12020–12027.
Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.
Kunkel, T. A., Roberts, J. D., & Zabour, R. A. (1987) *Methods Enzymol. 154*, 367–382.
Lange, P. H., Millan, J. L., Stigbrand, T., Vessella, R. L., Ruoslahti, E., & Fishman, W. H. (1982) *Cancer Res. 42*, 3244–3247.
Lehrach, H., Diamond, D., Wozney, J. M., & Boedtker, H. (1977) *Biochemistry 16*, 4743–4751.
Low, M. G., & Saltiel, A. R. (1988) *Science 239*, 268–275.
Machamer, C. E., Florkiewicz, R. Z., & Rose, J. K. (1985) *Mol. Cell. Biol. 5*, 3074–3083.
Martin, D., Tucker, D. F., Gorman, P., Sheer, D., Spurr, N. K., & Trowsdale, J. (1987) *Ann. Hum. Genet. 51*, 145–152.
Matzuk, M. M., & Boime, I. (1988) *J. Cell Biol. 106*, 1049–1059.
Matzuk, M. M., Keene, J. L., & Boime, I. (1989) *J. Biol. Chem. 264*, 2409–2414.
Micanovic, R., Bailey, C. A., Brink, L., Gerber, L., Pan, Y.-C. E., Hulmes, J. D., & Udenfriend, S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1398–1402.
Millan, J. L. (1986) *J. Biol. Chem. 261*, 3112–3115.
Millan, J. L. (1988) *Anticancer Res. 8*, 995–1004.
Millan, J. L., & Manes, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3024–3028.
Nakayama, T., Yoshida, M., & Kitamura, M. (1970) *Clin. Chim. Acta 30*, 546–548.
Ogata, S., Hayashi, Y., Takami, N., & Ikehara, Y. (1988) *J. Biol. Chem. 263*, 10489–10494.
Okayama, H., Kawaichi, M., Brownstein, M., Lee, F., Yokota, T., & Aria, K. (1987) *Methods Enzymol. 154*, 3–28.
Olden, K., Parent, J. B., & White, S. L. (1982) *Biochim. Biophys. Acta 650*, 209–232.
Olden, K., Bernard, B. A., Humphries, M. J., Yeo, T.-K., Yeo, K.-T., White, S. L., Newton, S. A., Bauer, H. C., & Parent, J. B. (1985) *Trends Biol. Sci. 10*, 78–82.
Ponte, P., Gunning, P., Blau, H., & Kedes, L. (1983) *Mol. Cell. Biol. 3*, 1783–1791.
Sakiyama, T., Robinson, J. C., & Chou, J. Y. (1978) *Arch. Biochem. Biophys. 191*, 782–791.
Sakiyama, T., Robinson, J. C., & Chou, J. Y. (1979) *J. Biol. Chem. 254*, 935–938.
Semenkovich, C. F., Luo, C.-C., Nakanishi, M. K., Chen, S.-H., Smith, L. C., & Chan, L. (1990) *J. Biol. Chem. 265*, 5429–5433.
Sowadski, J. M., Handschumacher, M. D., Kirshna Murthy, H. M., Foster, A., & Wyckoff, H. W. (1985) *J. Mol. Biol. 186*, 417–433.
Stigbrand, T., Millan, J. L., & Fishman, W. H. (1982) *Isoenzymes Curr. Top. Biol. Med. Res. 6*, 93–117.
Struck, D. K., & Lennarz, W. J. (1980) *J. Biol. Chem. 261*, 9721–9726.
Sussman, H. H. (1984) in *Human Alkaline Phosphatases* (Stigbrand, T., & Fishman, W. H., Eds.) pp 87–103, Alan R. Liss, Inc., New York.
Wahren, B., Holmgren, P. A., & Stigbrand, T. (1979) *Int. J. Cancer 24*, 749–753.
Watanabe, S., Watanabe, T., Li, W. B., Soong, B. W., & Chou, J. Y. (1989) *J. Biol. Chem. 264*, 12611–12619.
Whitaker, K. B., Byfield, P. G. H., & Moss, D. W. (1976) *Clin. Chim. Acta 71*, 285–291.
Wyckoff, H. W., Handschumacher, M., Murthy, H. M. K., & Sowadski, J. M. (1983) *Adv. Enzymol. 10*, 453–479.