MINIREVIEW

Alpha-fetoprotein Structure and Function: Relevance to Isoforms, Epitopes, and Conformational Variants

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Mammalian alpha-fetoprotein (AFP) is classified as a member of the albuminoid gene superfamily consisting of albumin, AFP, vitamin D (Gc) protein, and alpha-albumin. Molecular variants of AFP have long been reported in the biomedical literature. Early studies identified isoelectric pH isoforms and lectin-binding variants of AFP, which differed in their physicochemical properties, but not in amino acid composition. Genetic variants of AFP, differing in mRNA kilobase length, were later extensively described in rodent models during fetal/perinatal stages, carcinogenesis, and organ regeneration. With the advent of monoclonal antibodies in the early 1980s, multiple antigenic epitopes on native AFP were detected and categorized, culminating in the identification of six to seven major epitopes. During this period, various AFP-binding proteins and receptors were reported to inhibit certain AFP immunoreactions. Concomittantly, human and rodent AFP were cloned and the amino acid sequences of the translated proteins were divulged. Once the amino acid composition of the AFP molecule was known, enzymatic fragments could be identified and synthetic peptide segments synthesized. Following discovery of the molten globule form in 1981, the existence of transitory, intermediate forms of AFP were acknowledged and their physiological significance was realized. In the present review, the various isoforms and variants of AFP are discussed in light of their potential biological relevance. [Exp Biol Med Vol. 226(5):377–408, 2001]

Key words: alpha-fetoprotein; isoforms; epitopes; variants; conformation; structure; function

Alpha-fetoprotein (AFP), a tumor-associated fetal protein, has long been employed as a serum fetal defect/tumor marker to monitor distress/disease progression (1–3). Similar to albumin, serum AFP is known to bind and transport a multitude of ligands such as bilirubin, fatty acids, retinoids, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxin, and various drugs (4, 5). High concentrations of some of the hydrophobic ligands (i.e., fatty acids and estrogens) have been reported to induce a conformational change in the tertiary structure of AFP (see Ref. 6 for review). Altered serum AFP levels have been observed concurrent with aberrant growth manifestations, but it was usually assumed that these levels were a coincident effect rather than the cause of such changes. Although AFP may not be the direct cause of the altered growth manifestations observed in birth defects and cancer, it is conceivable that some shock/stress-induced conformational (variant) forms of this fetal protein may influence or contribute to such events. In the last decade, reports have emerged that some of these AFP forms may serve as dual regulators of growth, capable of both enhancement and inhibition (7, 8).

Molecular variants of mammalian AFP have been reported in the scientific literature since the 1970s. Initially, some of the variant forms were attributed to carbohydrate microheterogeneity and alterations in isoelectric points (9, 10). Additional AFP isoforms were genetic variants and lectin glycoforms that were detected and isolated by isoelectric focusing, electrophoresis, and chromatography (11, 12). Further isoforms were detected following high-pressure liquid chromatography (HPLC), and from lectin, heavy metal, and hydrophobic solid phase separation methods. The advent of monoclonal antibodies further permitted the

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detection and analysis of the epitopic domains and subdomains that comprise the total antigenic sites of this fetal protein (13, 14). Finally, the recent discovery and characterization of the molten globule form (MGF) have provided a new level of understanding regarding the various intermediate transition forms of proteins such as AFP (15).

Presently, there is no catalogue of the multiple isoforms and epitopic and conformational variants of AFP in the literature. Hence, the aims of the present review are 3-fold. First, the myriad of molecular forms of AFP found in human biological fluids and tissues, and in animal models, will be presented. Second, the various AFP isolates and isoforms that can be induced and purified in the laboratory from *in vivo* sources or from *in vitro* cell culture models will be described. Third, the relationship of AFP's biological activity to both naturally occurring and experimentally induced variant forms of AFP will be presented. The multiple names and classifications of the molecular variants of AFP in this rapidly advancing field justify a review that attempts to link structural findings with physiological effects. Finally, this review is intended to serve as an extension and update of a review on AFP as a biologic response modifier previously published in this journal (7). The present review is not intended to be all-inclusive for AFP, therefore the reader is directed to consult earlier reviews. (16, 17).

The Albuminoid Gene Family

AFP is classified as a member of an albuminoid gene family, which consists of four members to date: albumin (ALB), vitamin D-binding (Gc) protein (DBP), AFP, and alpha-ALB (αALB), termed afamin in humans (18, 19). This family is structurally characterized by cysteine residues that are folded into layers that form loops dictated by disulfide bridging, resulting in a triplet domain, U-shaped molecular structure (see Fig. 1). The three domains of these gene family members have been confirmed by X-ray crystallography (20, 21) (Figs. 1 and 2). The ALB gene family members display structural similarities, homologous amino acid sequence stretches, and similar cysteine disulfide bridge clusters (Fig. 1 and Table I). In humans, the four albuminoid genes lie in tandem on chromosome 4 within the 4q11-q22 region, encompassing 15 exons and 14 introns (22). DBP alone is truncated in the third domain (see Fig. 1) and contains only 13 exons, which results in a protein with a smaller molecular mass (23). The newest member of this gene family, α-ALB, was discovered in both rodent and human, and was cloned in the last 6 years (24, 25). All gene members are capable of ligand/carrier transport function, but display a vast array of other functions, including chemotaxis, oxygen free radical scavenging, esterase activity,

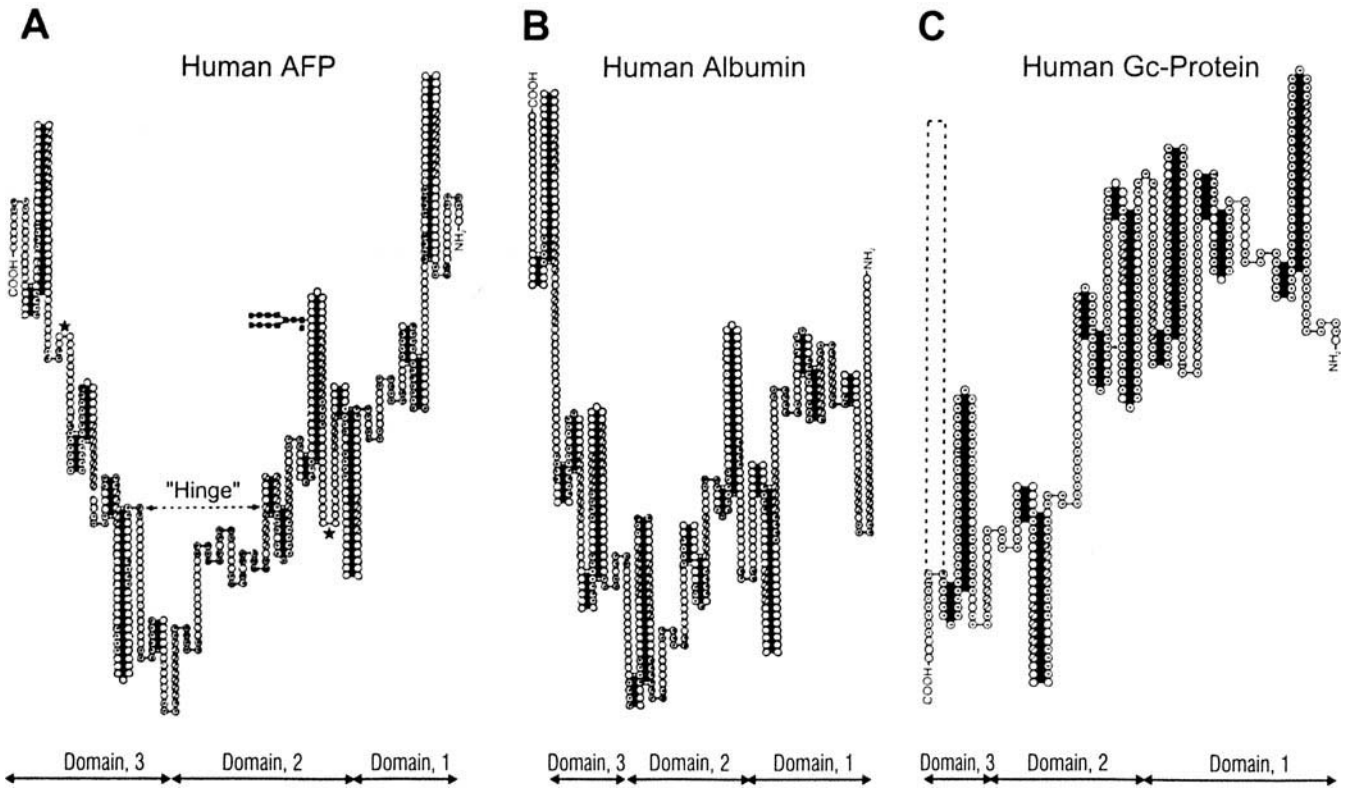

Figure 1. Molecular configurations of HAFP (left), human ALB (center), and human Gc DBP protein (right) based on the predicted secondary structures (See Ref. 117). Stars indicate predicted beta-turns and branched dark squares signify the sole carbohydrate side chain of HAFP. The dashed arrow indicates the proposed "hinge" region (lack of disulfide bridging) that is present in AFP, but absent in the other two gene family members. Note the truncated third domain of the Gc protein, representing a lack (dashed line) of a carboxy terminal amino acid chain compared with AFP and ALB. This figure was derived from composite diagrams redrawn from Refs. 117, 22, and 6.

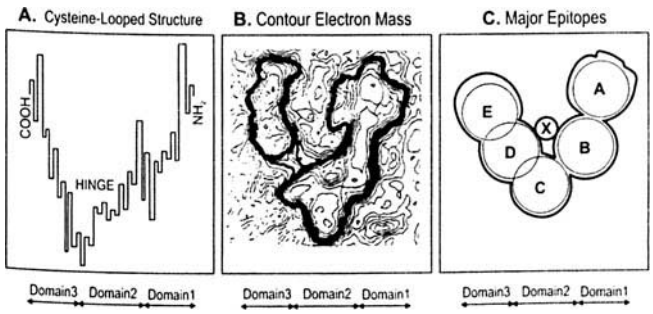

Figure 2. A three-panel diagrammatic mapping of the tri-domain structure of HAFP. (A) represents the cysteine-loop configuration, while (B) shows the electron dot contour mass map of HAFP. In comparison, (C) predicts an epitopic map of HAFP derived from 30 or more MoAbs (Refs. 14, 161). The precise epitope domain locations have been detected, but have yet to be localized. The three-panel diagram represents a composite redrawn from Refs. 6, 117, 21, 14, and 161.

leukocyte adherence, copper stimulated lipid peroxidation, and fatty acid, heavy metal, and actin binding, among others (26–28). Although the function of the recently discovered αALB remains obscure, it may play a role (ligand binding and immunoregulation) similar to its counterpart in lower vertebrates, a 74-kD ALB-like molecule found in fishes and amphibians (29, 30).

Genetic Variants. The genetic variants of mammalian AFP have been studied primarily in the rodent and to a lesser extent in humans (Table II). However, it is in mouse and rat that multiple gene expression has been described in greatest detail, including phase-specific expression of AFP mRNAs and their differential regulation during fetal and adult life, liver regeneration, and tumorogenesis (31–34). Although all of the AFP mRNA variants have been translated into proteins *in vitro*, not all have been detected *in vivo*. The major fetal- and tumor-derived AFP mRNA consists of a 2.2 -kb transcript that translates to a 69,000 to 73,000 molecular form in human and rodents, depending on its carbohydrate content (35, 36). The fetal rat liver primarily expresses the 2.2-kb transcript, but produces a 1.7-kb moiety that translates into a 50- to 65-kD protein that appears in the last quarter of the rat gestation period (37, 39) (Table II). In the neonatal rat liver, a 1.5-kb message (in addition to the 2.2 and 1.7 kb) is detected, which gives rise to a 48-kD protein present for only 4 to 8 weeks following birth. In contrast, the adult liver AFP mRNA transcripts contain only traces of the 2.2-kb moiety with low amounts of the 1.7- and 1.5-kb transcripts and a more recently detected 1.35-kb transcript (37 kD)(39, 40). This latter 1.35-kb form lacks one-third of the amino terminal first-domain of rat AFP (41, 42). Finally, the hepatoma and liver regeneration forms of rat AFP display largely the 2.2-kb form and lesser amounts of the 1.7-kb variant (35, 36).

The 1.35-kb AFP mRNA found in adult rat liver and kidney deserves special mention in that a similar transcript has been detected in human embryonal carcinomas transplanted into nude mice (43). In both species, a 1.35-kb form is retained intracellularly as a non-secreted form first reported in uterine and cancer cells (44, 45). This variant represented only 40% of the normal human AFP molecule translated from the 2.2-kb mRNA; it lacked the entire first domain and one-third of the second domain. It may be of interest that orphan steroid receptors, which dimerize with other nuclear receptors, have a similar truncated structure (46–48). Thus, truncated AFP forms could potentially bind to steroid receptors as previously proposed (49).

The adult form of human AFP, like the rat form, might also be derived from multiple RNA transcripts (i.e., 2.2, 1.7, and 1.6 kb). It is well established that the human form of AFP detected in most radioimmunoassays (RIAs) and enzyme-immunoassays (EIAs) represents the 69,000- to 70,000-kD (2.2 kb) polypeptide. It is highly probable that other AFP mRNA transcripts are present and are translated in serum and/or tissues, but are undetectable by present immunologic assays. Isolation and characterization of these predicted forms would be even more difficult since their concentrations would be vanishingly small (i.e., nanogram to picogram levels).

An interesting composite can be drawn when the genetic variants of the rat, and presumably human, are considered. As discussed above, the genetic variants of rat AFP mRNA consist of sizes ranging from 2.2 to 1.35 kb, representing translated proteins ranging from 72 kD down to 37 kD, respectively (41, 42). The smaller protein forms are found to be truncated from the amino-terminal end. This indicates that truncation eliminates domain 1 (amino-

Table I. Biochemical and Structural Properties of the Four Known Protein Members of the Human Albuminoid Gene Family

Member name (protein)	Molecular weight (kD)	Sediment coefficient (s)	N-Glys sites (n)	Carbohydrate content (%)	Peptide domains	Total amino acids	Messenger RNA (kb)	Chromosome location	Organ of synthesis	Serum concentration (μg/ml)
Serum albumin	66	4.5	0	0.5	3	585	2.1	4	Adult liver	30,000
Alpha-albumin	82	5.3	4	21.0	3	608	2.3	4	Adult liver	20
Alpha-fetoprotein	69	4.5	1	3.0–5.0	3	590	2.2	4	Fetal liver and yolk sac, (newborn liver)	3,000 (50)
Vit-D-binding protein (GC)	58	4.1	1	5.0	2.6	458	1.7	4	Adult liver	400

Note. N-Glys, N-linked glycosylation sites. Parentheses indicates a quantitative difference in liver secretion. Data compiled from Refs. 5, 6, 7, and 63.

Table II. Expression Patterns[a] of Encoded Rat Alpha-Fetoprotein (AFP) mRNAs and Their Translated Proteins During Developmental and Adult Stages

Rat AFP mRNA and their translated protein variants					
MRNA (kb)	2.2	1.7	1.5	1.35	References
Translated protein (kD)	73, 69	65, 59	50, 48	44, 37	34–42
Development stages	Presence or absence of expression of variants				
Fetal	++	+	+	–	37, 38, 42
Perinatal	++	+	–	–	38, 39
Postnatal	+	+	+	–	39, 34
Adult	±	+	+	+	40, 41
Cancer presence	++	+	–	+	35, 36, 43
Regeneration	++	+	–	–	34, 36, 39

Note. The trend of reduced presence of the 2.2-kb form as development to the adult proceeds (read down the column) and in reduced size of the translated message at the perinatal and adult stages. ++, copious amounts; +, moderate amounts; ±, trace amounts; and –, none detected.

[a] Presence (+) or absence (–) of expression.

terminus) and a portion (up to one-third) of domain 2 of the rat AFP molecule as in the extreme case of the 1.35-kb form. As demonstrated in a previous phylogenetic review of AFP (6), it is the amino-terminal portion (domain 1) that distinguishes rat AFP from other mammalian forms (i.e., human AFP). In that review it was shown that domain 3 exhibits greater amino acid sequence identity among the various mammals than do domains 1 and 2. Domain 3 is also known to contain a major hydrophobic-binding site on human (H) AFP and a proposed dimerization motif (7, 49), now supported by experimental evidence (50). Domains 1 and 2 contain binding sites for both fatty acids and bilirubin; however, domain 2 also displays amino acid sequences related to cellular and extracellular matrix (ECM) adherence regions and bears sites for the carbohydrate attachment via asparagine. It may be more than coincidence that the truncated 1.35 kb (third domain) is retained within the cell as a nonsecreting form, since it bears no carbohydrate side chains (for blood circulation) and might be capable of heterodimerization to other intracellular proteins as do the truncated steroid nuclear receptors (47, 48). It is germane to this discussion that most commercial companies utilize as their capture antibody, monoclonal antibodies (MoAbs) directed to the first domain of AFP, and use for detection labeled secondary antibodies to the less specific second and/or third domain of AFP. If this is the case, most assay kits would contain antibodies directed largely against the 2.2-kb translated protein (72 kD); hence, the other truncated forms would never be detected.

Regulation and Expression. The genetic regulation and expression of mammalian AFP is a highly complex area of study, and space does not permit adequate coverage in the present review. The reader is referred to recent reviews by Chiu and by Lazarevich (51, 52) for excellent detailed descriptions and overviews of this exciting field. Other papers using derivatives of DNA or mRNA for AFP as a genetic vector, as antisense, and as mRNA and cDNA isolates will only be briefly mentioned. For example, the presence or absence of HAFP mRNA in peripheral blood has been employed as a predictor of outcome in patients with hepatocellular carcinoma (53). Another study employed the AFP gene promoter for gene therapy against high AFP-secreting hepatoma cells using a retrovirus vector carrying a herpes simplex thymidine kinase gene (54). These same investigators then used a variant of the AFP gene promoter for gene therapy against a low AFP-producing hepatoma (55). In other studies, an AFP antisense strategy was employed to inhibit the growth of human liver cells in culture (56). Two further genetic immunization schemes were developed using AFP as a target for T-cell immune responses. In one study, dendritic cells were engineered to express AFP-produced potent AFP-specific cytotoxic T-lymphocytes directed against hepatoma cells (57, 58). Studies were also reported in which HAFP was employed as a recombinant reporter to target proteins to the plasma membrane of cells (59). Potential uses of this technique may include vaccine development, tissue engineering, genetic research, bioseparations, and disease treatment. This latter study served to show that high levels of monomeric and dimeric proteins could be targeted to the cell membrane by the proper selection of a transmembrane domain attached to a reporter protein (AFP). Also, reports of additional AFP gene promoters and repression of the AFP gene continue to emerge (60–62). Finally, an AFP transcription factor has also been described that is a nuclear receptor related to the Drosophila FTZ-F1 gene family (326).

Free and Bound Molecular Forms

As stated above, many genetic variants of AFP mRNA have been reported in both human and rodent. Some of these translated AFP products are present in both bound and free forms. The reader is directed to previous reviews by the author, which detail the various bound forms of mammalian AFP in both serum and tissues (7, 63). Although elusive and difficult to detect, AFP complexed to binding proteins has a documented history dating back to the original observation by Norgaard-Petersen in 1976 (64). Since that time, sufficient articles describing AFP-binding proteins and receptors

have been reported to validate their existence and justify their place in the scientific literature (65–69).

Soluble Forms. Some of the bound forms of AFP in solution can be detected directly by immunological methods, while others can only be observed following use of physical separation solutions such as 0.4 M KCl or even more harsh organic chemical exposure (urea, guanidine-HCl, etc.). Some of the reported candidates for AFP-binding proteins include IgG, IgM, actin, TGF-β, osteonectin, and protease substrates and inhibitors (reviewed in Ref. 7). It has also been well documented that AFP undergoes self-aggregation to form dimers, trimers, and oligomers (70, 71) *in vitro,* and at least dimers have been detected *in vivo* (72). The latter investigators (*in vivo* study) also reported that the dimeric form of AFP may contribute to its function regarding E_2-stimulated growth.

Membrane Receptors. Four cell surface receptors for AFP have been described that might bind various forms of AFP in addition to the native protein. These receptors have been described as both endothelial components and epithelial cell surface membrane receptors (73–76). Eighteen-, 31-, and 60-kD cell surface receptors (73) have been found in the vascular endothelium of many tissues (heart, lung, epididymus, etc.). The 18- and 31-kD binding proteins represent scavenger receptors that bind chemically modified or denatured albumin and AFP, while the 60-kD form is an endothelial cell surface sialoglycoprotein found only on continuously lined (not sinusoidal) endothelium. However, a canonical AFP cell surface receptor has been localized on monocytic, reproductive, immunologic, and tumor cells, notably, hepatomas and MCF-7 breast cancer cells (74–76, 338). This AFP receptor is a 62- to 67-kD protein first detected on human breast cancer cells (77) and later purified from monocyte cell membrane preparations (74). In summary, the field of AFP-binding proteins and receptors is in the early stages of discovery and these receptors have yet to be scrutinized and subjected to cloning, chemical characterization, and physiological study.

AFP Fragments

Plasma proteins such as ALB and AFP could possibly serve as circulating protein reservoirs of biological response-modifying peptide fragments. Many proteins are known to serve as precursor molecules and to contain multiple modular sequences or cassette segments generated by proteolytic processing to produce smaller biologically active peptides (78–81). Limited proteolysis of larger proteins appears to be a general mechanism for *in situ* generation of a variety of regulatory peptides in the circulation (clotting, bleeding, and complement fixation) (69). In many cases, a simple, large polypeptide serves as the precursor proprotein (pre-proprotein) for a host of biologically active peptides that function in the gastrointestinal, endocrine, cardiovascular, and nervous systems. The peptidic and short amino acid segments (fragments) cleaved from the substrate often display biological effects that differ from the "parent" protein. One such example would be the angiogenic peptides developed by Judah Folkman *et al.* (82) such as angiostatin and endostatin, which are fragments derived from collagen and plasminogen, respectively. Endostatin, for example, is cleaved from the collagen protein by elastase (83). Such peptidic fragments may participate in a host of biologic roles, including biochemical antagonism and agonism, hemostasis, feedback control, and hormonal modulation/regulation. It has been previously reported that short amino acid sequences of ALB and AFP demonstrate sequence similarity with cleaved fragments from neurotensin and neuromedin (84). These peptides were cleaved from a kinetensin "parent" protein, which generated peptides capable of modulating biological responses in the endocrine, cardiovascular, digestive, reticuloendothelial, and central nervous systems (see below and Table III). It is of interest in this discussion that excessive placental secretions of neurokinin have recently been associated with pre-eclampsia in the third trimester of pregnancy (333). It has long been known that pre-eclampsia is associated with elevated AFP levels (334, 335).

Enzymatic Fragments. The ALB molecule has been repeatedly subjected to enzymatic digestion using trypsin, pepsin, chymotrypsin, and others to determine ligand-binding sites (85). In contrast, few studies employing enzymatic digestion of AFP have been reported, other than those using mass spectrometric analysis of the fetal protein (86). However, a recent study by Dudich *et al.* (50) subjected HAFP to time-limited (mild) peptic hydrolysis using charcoal stripped ligand-free preparations. These investigators obtained and characterized two major AFP peptide fragments, 38 and 32 kD, which were further hydrolyzed to two proteolytic resistant moieties of 23 and 26 kD, respectively. These latter two fragments retained reactive secondary, tertiary, and antigenic structure and were representative of the compact, rigid forms of domains 1 and 3. In contrast, the chemical behavior of the interconnecting domain 2 demonstrated a secondary structure, but lacked a rigid tertiary structure, a form consistent with the "molten globule" state. When subjected to tests of biological activity employing apoptosis, it was determined that both P23 and P26 (domains 1 and 3) in a dimer state, together with participation of domain 2 were required for apoptotic signaling in a Raji cell line. Both full-length AFP and its fragments have been implicated in both apoptosis and tumor cytotoxity.

Synthetic Peptide Fragments. The first biologically active synthetic peptide derived exclusively from HAFP was reported by the author's laboratory for a segment that regulated estrogen-induced growth (87). In that report, an amino acid segment from HAFP 445 to 480 was synthesized by F-Moc chemistry, purified by reverse phase (C-18) HPLC, and structure-confirmed by electrospray mass spectroscopy and amino acid sequence analysis. Subjection to circular dichroic spectroscopy and Fourier infra-red spectroscopy revealed a secondary structure consisting of 10% alpha-helix, 50% beta sheets and turns, and 40% random

Table III. Human Alpha-Fetoprotein (HAFP)-Derived Peptides Listed According to Domain, Amino Acid Location, and Proposed/Observed Functional Activity

Peptide name	Amino acid[a] sequence/number	Domain location	Functional activity	References
38-kD	~1–250	1	Apoptosis related peptide	50, 284
23-kD	~1–50	1		
32-kD	~400–590	3	Apoptosis-related polypeptide	50, 289
26-kD	~500–590	3		
Predicted leucine heptad repeats	496–533 534–588	3	Protein-to-protein interaction site	49
Kinetensin-like segments	146 IARRHPFLY 339 MSRRHPQLA 536 LVKQKPQIT	1 2 3	Neurotensin-related cardiovascular events	7, 84
Growth inhibitory peptide (P149)	447 LSEDKLLACGEG 459 AADIIIGHLCIRH 472 EMTPVNPG	3 3 3	E_2 Receptor-Binding E_2-Binding Non-E_2 Growth Inhibitor	63, 30
Insulin-like segment	13 LDSYQC	1	Glucose uptake mediator	88
Epidermal growth factor-like segment	13 LDSYQCT	1	Overcoming drug resistance	89, 90
Histocompatability segment	522 GVALQTMKQ	3	HLA-class-I peptide	57, 58
Extracellular matrix motifs	252 RGD 194 LRE	2 1	Cellular adhesion tripeptide	7, 97
Major fatty acid-binding site	209 KNFGTRTFQAIT VTKLSQK	2	Fatty acid-binding interface	116, 139
Estrogen-binding site (rodent)	423 APELIDLTGKMVS IASTCCLSEE	3	E_2- and E_1-binding (mouse) interface	314, 315
Predicted actin-binding site	375 DKGEEELQ	2	Possible actin-binding interface	7, 109
Human milk peptide segment	398 GLF	2	Binds to macrophages	7, 316
Predicted Zn^{++}-binding site	244 VAHVHEHC	2	Possible heavy metal-binding site	312, 313
Proline-rich motif	AAA: 110, KKPTPASIPL-FQVALPEPV	1	Possible SH3 ligand	7, 317
Plasminogen activator-like segment	552–590	3	Growth regulation of transformed cells	7, 79
Segment lacking ALB identity	38–119	1	HAFP-specific monoclonals	6
Segments showing ALB identity	145–160 488–504	2 3	HAFP receptor crossreactivity	311

Note. HLA = histocompatability locus; SH3 = SHr protein-to-protein interaction.
[a] Amino acid single letter code.

coil. The peptide, numerically designated synthesis No. P149, was capable of inhibiting both steroid-dependent and -independent growth events, including thyroid-induced frog metamorphosis (30). Computer modeling of the P149 peptide revealed a largely hydrophilic linear peptide with a right angle beta-turn in the last third of the carboxy-terminus. Subsequent studies have shown that the peptide was capable of growth suppression in both estrogen-dependent and -independent tumors (173).

The use of other AFP-derived peptides has increased in the last 5 years (Table III and Fig. 3). These studies have involved peptides located near the carboxy-terminus of domain 3 and some located near the amino-terminal side of domain 1. A synthetic peptide from domain 1, LDSYQC, was studied to determine its influence on glucose uptake by human erythrocytes (RBCs). The LDSYQC peptide was found to stimulate the entry of glucose into RBCs from insulin-dependent diabetic children after a 1-hr treatment in 10^{-8} M to 10^{-6} M peptide *in vitro* (88). It was of interest that the peptide effect mimicked that of insulin at 10^{-9} M to 10^{-7} M concentrations. An amino acid sequence in insulin (amino acids 17–21) was found to be homologous to the AFP peptide. It was this same sequence on AFP (LDSYQCT) that also increased the antiproliferative potency of the drug "Cytozar" on cultured lymphocytes from chronic lymphoid leukemia patients (89). The LDSYQCT peptide at 10^{-8} M to 10^{-7} M significantly increased the suppression of lymphocyte proliferation in cells from patients experiencing drug resistance. Further, sequence matching of the AFP amino acids 13 to 19 by these investigators showed amino acid identity/similarity not only to the insulin alpha-chain (see above), but also to epidermal growth factor (amino acids 26–32) and glycodelin (amino acids 67–67 and 114–120) (See Ref. 90).

An AFP-derived peptide has also been identified as a potential HLA-A2.1-restricted peptide epitope obtained from a computer analysis of the HAFP complete sequence. Amino acid sequence 542 to 550 from the third domain of HAFP (GVALQTMKQ) was found to bind with low affinity to CD8 dendritic T-cells bearing HLA-A2.1 class I alleles, and displayed slow dissociation kinetics (58). The No. 542 to 550 HAFP peptide generated the induction of human lymphocyte T-cell clones in culture; this peptide in transgenic mice recognized AFP-transfected targets in both cy-

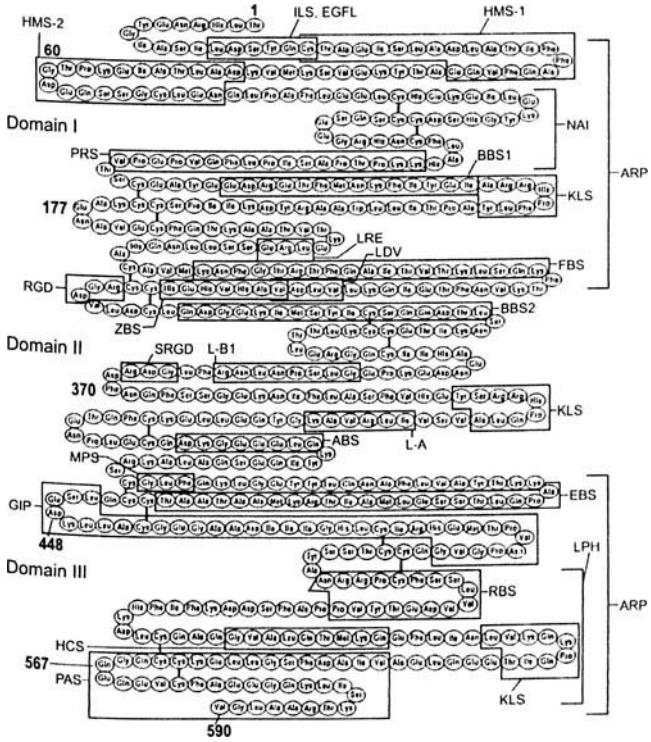

Figure 3. Amino acid peptide sequences of HAFP with known or proposed biological (physiological) activities. Sequences include both enzyme-derived fragments and synthetic segments. ABS, (proposed) actin-binding site; ARP, apoptosis-related peptide; BBS 1,2, (predicted) bilirubin-binding sites; EBS, (documented) estrogen-binding site (in rodents); EGFL, epidermal growth factor-like segment; FBS, (confirmed) fatty acid-binding site; GIP, (documented) growth inhibitory peptide; HCS, histocompatability Class II (confirmed) segment; HMS 1,2, (predicted) heavy metal-binding sites; ILS, (GenBank derived) insulin-like segment; KLS, kinesin-like segments; LPH, leucine predicted heptads; L-A, (GenBank derived) laminin-A segment; L-B1, laminin-B1 segment; LRE, LDV, and RGD, (documented) cell adhesion sequences (one-letter amino acid code); MPS, (documented) milk casein peptide segment; NAI, nonALB identity site (confirmed); PAS, (GenBank derived) plasminogen activator segment; PRS, proline-rich sequence; RBS, (proposed) HAFP receptor-binding site; SRGD, segment reversed RGD site; ZBS, (proposed) zinc-binding site. Data obtained from Refs. 7, 49, 63, 87 through 94, 96, and 97.

totoxicity and cytokine release assays. These studies demonstrated that AFP-reactive clones have not been deleted from the human T-cell repertoire, and that AFP is a potential target for T-cell-based immunotherapy using peptide-based specific strategies.

Cellular Adhesion Sequences

The present discussion concerns features unique to the second domain of AFP, which has short peptide sequences common to ECM proteins bearing cellular adhesion motifs (CAMs). This finding distinguishes AFP from ALB, αALB, and DBP, which do not include short peptide sequence similarities to the ECM protein family (laminin, fibronectin, collagen, vitronectin, thrombospondin, etc.) of adhesion macromolecules (91). Adhesive macromolecules have potential utility in developmental and disease states involving growth, differentiation, cell migration, and tumor metastasis. Using synthetic peptides derived from the ECMs, the functional significance of such short signal peptide sequences has been identified from one or more domains of these molecules (92–94). Some of the CAM-derived synthetic peptides have been found to block cell differentiation, tumor growth, and angiogenesis. Some ECM proteins (laminin, collagen, and fibronectin) contain multiple biologically active peptide sequences with differing activities specific to cell type (95). Also, the various cellular receptors for a particular active site sequence may differ slightly among specific cells, permitting a large diversity of biological functions and cell regulatory roles. Mammalian AFP, especially the second domain, contains short peptide sequences that are common to the ECM proteins. Further, AFP appears to possess a multiplicity of such peptide sequences, which suggests involvement in a diverse range of biological activities.

A variety of cell attachment peptide sequence sites from ECM proteins (see Refs. 91–108) have been listed in Table IV (left column) together with mouse AFP, and HAFP, ALB, α ALB, and DBP numbered peptide sequences. On inspection of the matched peptide sequences, it is observed that frequent amino acid matches of AFP with CAM-like sequences occur in the second domain of AFP (amino acids 190–394). Exact amino acid matches appear less frequently on human ALB and DBP molecules. The physiological functions of the adhesion sequences of the CAMs and the cell types involved are listed in the right hand column of Table IV. It becomes apparent that a large number of diverse cellular adhesion activities might be involved and that AFP shares short sequence similarities with a variety of the different CAM segments identified to date. Such a diverse array of peptide recognition signals suggests that AFP might share functional properties with adhesion molecules, as previously suggested (7). However, such capabilities of AFP might ultimately depend on its ligand-bound state and extracellular localization during growth.

It is of special interest that a periodic spacing of the signal recognition peptides is readily observed in the second domain of AFP. The first of the known peptide signals, LRE, is positioned at the amino-terminal side of domain two on the HAFP molecule (amino acids 194–196). There follows a gap of approximately 40 amino acids before a second signal peptide (LDV) occurs at HAFP amino acids 241 to 243. It is at this point that a pattern of regularity arises. The next four signals (including LDV) occur approximately every 10 amino acid sequences from amino acids 240 to 270, including LDV (fibronectin), RGD (fibronectin), DGEK (collagen I, IV), and YICSQ (laminin B1). The RGD sequence on AFP may be positioned on a tight turn of an exposed loop, as seen on the crystal structure of ALB, which is similar to AFP (20) (Fig. 2). The next proposed signal peptide at AFP amino acid 309 (PNLDR) is similar to laminin B1, followed by a scrambled RGD at amino acid 316, and a sequence at amino acid 351 (ILRVK) comparable with laminin-A. Scrambled RGD is known to be a growth inhibitor sequence (97).

Table IV. Cell Adhesion Sequences in Extracellular Matrix Proteins Compared With Sequences on Alpha-Fetoprotein, Albumin, Vitamin-D-Binding Protein, and Alpha-Albumin

Molecule and sequence number	Amino acid* recognition sequences	Proposed action	Reference
Laminin-S	L R E	Promotes adhesion of ciliary ganglia	91
HAFP 194	L R E		
MAFP 189	L R E		
HALB 189	L R D		
HDBP 184	L K E		
Fibronectin	L D V	Promotes cell adhesion and spreading in neural crest cells and lymphocytes	99, 100
HAFP 241	L D V		
MAFP 234	L D V		
HALB 235	T D L		
HDBP 233	E D V		
Fibronectin	R G D	Promotes cell adhesion, inhibits tumor metastases, reduces fibrinogen-platelet aggregation and affects differentiation angiogenesis	97, 98, 100, 106, 105
HAFP 252	C R G D		
MAFP 247	C Q G D		
HALB 247	C H G D		
HDBP 251	C G S A		
Collagen I and IV	D G E A	Blocks platelet adhesion to collagen, but not fibronectin and laminin and blocks cancer cell adhesion to collagen	110, 111
HAFP 261	D G E K		
MAFP 256	D G E K		
HALB 256	D R A D		
HDBP 257	D C M A		
Laminin B1	Y I G S R	Not reactive with neural cells, inhibits tumor metastases (growth and angiogenesis), and promotes cell migration	93, 95, 103
HAFP 268	Y I C S Q		
MAFP 263	Y I C S Q		
HALB 263	Y I C S Q		
HDBP 268	K L C D N		
Laminin B1	P D S G R	Inhibits tumor growth and metastases, angiogenesis on chorio-allantoic membranes	108
HAFP 309	P N L N R		
MAFP 304	P S Q F L		
HALB 303	P S L A A		
HDBP 305	P E L P D		
Scrambled RGD	L G D R	RGD inhibitor/antagonist; MHC Class II-related	97
HAFP 316	L G D R		
MAFP 311	L G D R		
HALB 311	V E S K		
HDBP 314	P T N K		
HAAB 308	R E G K		
Laminin A	S I K V A V	Reactive with neural cells and increases tumor metastasis, plasminogen activation, and collagenase activity	92
HAFP 351	I L R V A K		
MAFP 345	I L A V S V		
HALB 346	L L R L A K		
HDBP 345	L S K V L E		
HAAB 355	L L R I V Q		

Note. Letters indicate the single-letter amino acid code. HAFP, human alpha-fetoprotein; MAFP, mouse alpha-fetoprotein; HALB, human serum albumin; HDBP, human vitamin-binding protein; and HAAB, human alpha-albumin.

Functional Significance of CAMs. Functional subdomains of the CAMs have been described and their biological significance has been realized and delineated (9, 13, 28, 91). Multiple studies of CAM recognition signals have shown that they modulate an array of critical biological activities (See Refs. 98–105). Some of these activities include cell adhesion, migration, differentiation, growth, neurite outgrowth, tumor spread, enzyme activity, angiogenesis, and heparin, fibrin, collagen interaction (Table IV). The CAMs located in the ECM interact with a vast array of cell/tissue types, which include epithelial, muscle, fat, peripheral neural, fibrous connective, platelet, endothelial, and tumor cells (melanoma and adenocarcinoma cells) (91). This interaction involves binding of the CAM to cell surface integrins.

The native adhesion proteins (i.e., laminin, collagen, fibronectin, etc.) are found primarily in basement membranes, often tethered to collagen and fibrillar networks. Since each of these proteins contains multiple biologically active adhesion signal peptides, they collectively exert a growth regulatory function on cell migration, spreading, and differentiation. Modulation at these control levels is also a crucial factor in tumor growth and metastasis. Some adhesion proteins are known to contain two signals of opposing activities (YIGSR and SIKVAV of laminin), demonstrating that additional fine-tuning controls are in place on the same

molecule to even further regulate adhesion activities (106). Two such sites may not be simultaneously available on a protein due to ligand and/or conformational masking and could be progressively unveiled during a cascade such as the clotting/anticlotting network. Although the integrin cell receptors for a particular adhesion peptide are distinct for specific cell types, it is known that some integrins can recognize multiple sites on CAMs such as the YIGSR, RGD, and SIKVAV sequences for laminin (96).

Cell Adhesion During Development. These adhesion peptides have also been studied during various developmental stages and have been shown to influence cell differentiation, tissue elongation, cell migration of mesenchyme and neural crest cells, and specific stages of embryonic development (100, 107). Specific peptide sequences (YIGSR) can determine basement membrane cellular attachment, Sertoli cell cord formation, endothelial and salivary cell alignment on basement membranes (SIKVAV), cell adhesion and spreading, and angiogenesis on chorio-allantoic membranes (100, 108). In neural growth, active synthetic peptides can regulate neurite outgrowth, promote the adhesion of ciliary ganglia, recognize various neuronal cells, and influence tyrosine hydroxylase activity in neural transmitter precursors (96). In cancer growth, active site peptides can block adenocarcinoma cell adhesion to collagen and/or laminin or enhance melanoma cell adhesion to such proteins (100, 101). Finally, in hemostasis, adhesion peptides can regulate angiogenesis, plasminogen activation, collagenase IV activity, platelet adhesion, and sites for heparin binding. Most cells use the various integrin-receptor systems as a basis for such activities (109, 110). These data suggest that AFP might be capable of binding to the integrin-receptor system and should prompt further binding studies (111).

Proposed Significance for AFP. From the teleologic standpoint, it would be reasonable that a fetal protein with reported growth regulatory activities would display an array of adhesion peptides sites in its primary structure (See review in Ref. 111). It would be logical that a serum fetal protein, which ontogentically precedes ALB formation and synthesis, could possess an extensive armamentarium of adhesion site signals that might influence growth and cell differentiation. However, the similarity on AFP of such a diverse multitude of sequence signals common to a variety of adhesion macromolecules may be more than coincidence. This suggests that AFP may play a role in fine-tuning the architectural interstitial growth patterns in developing organisms.

Following the aforementioned logic, it appears that AFP could be armed with pairs of adhesion peptide signal sequences, some of which are known to function with opposing activities. Thus, AFP might possess an array of peptide sequences that differ slightly from the CAM sequence and thus serve as inhibitor sequences (see Table I, GDR versus RGD). As alluded to above, all adhesion sites may not be concurrently accessible since AFP is thought to express different functions at various developmental stages (4). Some sites may be unmasked or made conformationally available during progressive stages of embryonic and fetal development. As in the case of RGD, it appears that the peptide sequences and tertiary structure surrounding that particular peptide site could be important for its eventual activity or its competition in the native AFP molecule, as was shown for the cyclic peptide CRGDCL (112). It is also known that several upstream (amino terminal) sequences in fibronectin function as synergistic cohorts regarding RGD activities (113, 114). It is of interest that AFP binding of fatty acids, bilirubin, and various drugs occurs in the second domain of the molecule (7). In fact, fatty acids are known to be bound at amino acids 209 to 227, in a region lacking CAM sites (115, 116).

The proposed activity of these adhesion peptides in AFP must of course await experimental verification. The peptides are easily synthesized; however, suitable experimental models to assay their biological activity are not as readily available. Such cell adhesion models do exist and efforts to document their activities might prove rewarding for therapeutic and diagnostic purposes. Undoubtedly, there are additional active peptide sequences in AFP, especially in the non-CAM peptide region (amino acids 197–240) described above or in other domains. The use of synthetic AFP-associated CAM peptides could more clearly delineate and possibly unravel developmental mechanisms concerning growth, differentiation, and tissue architecture. Such AFP peptides may find utility as therapeutic, diagnostic, or screening (marker) agents in diseases such as cancer and hematological disorders. Others might find uses in biotechnology procedures such as cell cloning and hybridization, matrigel cell culture assays, coating of artificial transplants/implants, and adsorption to prosthetic devices (91). Finally, it is conceivable that cells or proteins armed with adhesion peptides could be targeted to areas of chronic or acute inflammation to possibly accelerate wound healing, inhibit metastases, and minimize the formation of scar tissue at lesion sites.

Molecular Microheterogeneity

Carbohydrate Isoforms. In contrast to ALB, AFPs are glycoproteins containing carbohydrate moieties in one to three N-linked glycans. While HAFP contains only one N-linked glycan at Asn 232 on domain 2, mouse AFP displays three sites at Asn 232, Asn-310 on domain 2, and Thr 483 on domain 3, and rat AFP possesses three sites at Asn 232, Ser 96 (domain 1), and Asn 310 on domain 2 (117, 6, 7). In human, the structure of the glycan on AFP produced by a hepatoma differed from that produced by a yolk sac tumor (11). The AFPs produced in cells from different organs and under various pathological conditions have the same sequence and are immunologically the same (2.2-kb form); thus microheterogeneity is displayed by AFP's reaction to different lectins and to pH environments (see below).

Different isoforms can be demonstrated by electropho-

retic, chromatographic, and isoelectric techniques in combination with methods employing lectins (See Refs. 118–126). Therefore, most researchers denote AFP variants as binding (reactive) or not binding (nonreactive) with their respective lectins (Table V). The three lectins that have been most useful for studies of AFP are concanavalin-A (Con A), *Lens culinaris* agglutinin (LCA), and *Vicia faba* agglutinin (VFA) with a d-mannose specificity (11, 118). Lectin specificity for d-galactose is also demonstrable with *Ricinus communis* agglutinin-1 (RCA), and *Viscum album* agglutinin-1 (VAA), both of which react and bind to the carbohydrates of HAFP. The galactose-specific lectins are thought to react with the penultimate galactose of the biantennary AFP glycan chain. The lack of reactivity with other lectins permits one to determine the structure of the glycan of all AFP variants (126, 127). However, the mannose-specific lectins require the presence of one fucose bound to the glycosyl-N-acetyl (GlcNAc) next to the Asn residue. Studies have shown that most molecules of HAFP possess a biantennary glycan substituted with two sialic acid residues (11). Some lectins such as phytohemagglutinin can be induced to bind to HAFP following removal of sialic acid residues (123). Wheat germ agglutinin, which does not bind to HAFP, requires an additional fucose adjacent to the GlcNAc next to the Asn residue (125).

The sugar chains are not genetically encoded, but are dependent on the set of glycosylation enzymes present in the endoplasmic reticulum (ER) and the Golgi complex of the host cell. However, these enzymes have different tissue distributions, and the variants of AFP are due to the various tissue specific pathways involved (128–131). Thus, yolk sac-derived AFP differs from AFP of hepatic origin. However, AFP in amniotic fluid consists of a mixture of these two types in different ratios, depending on gestational age. Differences encountered in various tumor types also suggest quantitative as well as qualitative differences in the glycosylation enzymes from each tumor (Table V).

The carbohydrate heterogeneity of HAFP has spurred the use of lectin-affinity separation techniques for use in the diagnosis of specific fetal defects and for distinguishing between different tumor types. It was first shown in 1979 that isoforms in amniotic fluid AFP contained Con-A-binding and Con-A-nonbinding variants as a mixture from both yolk sac- and liver-derived origins (128). The nonbinding variant is decreased in pregnancies with neural tube defects, and can be separated and quantitated by column chromatography. By 1981 it was already obvious that determination of AFP-branched sugar heterogeneity would be a useful tool in the differential diagnosis of cancer (132, 133). Yolk sac tumors and gastrointestinal cancers could be distinguished from hepatomas based on LCA reactivity, and from benign liver disorders (nonreactive LCA) employing imaging procedures (134). The addition of lectin affinity chromatography and affinity electrophoresis in conjunction with autoradiography, indirect enzyme-linked immunoabsorbant assay (ELISA), and blotting techniques has since amplified the detection of a multitude of AFP glycoforms. These techniques have added a new dimension to identification since variants can be detected not only as binding and nonbinding bands, but also as gel retardation moieties.

Using combined lectin affinity-based procedures, i.e., crossed affinity immunoelectrophoresis, at least 10 HAFP glycoforms have now been identified and characterized (126). Lectins have been found to be specific not only for sugar residues, but also for the whole carbohydrate molecule. More recently, inclusion of additional lectins such as

Table V. Specificity Reaction or Nonreaction of Selected Lectins With Human Alpha-Fetoprotein (HAFP) IsoForms and Their Clinical Utility as Biomarkers in the Differential Diagnosis of Cancer and Fetal Defects

Type of lectin	HAFP reactivity	Sugar specificity	Isoforms detected	Clinical utility	Reference
Concanavalin A	Reactive	d-mannose	2	PRG, HCC, YCT, MHC	118, 119
Lens culinaris agglutinin	Reactive	d-mannose (l-fucose)	2	HCC, LCH, BLC, MHC	134, 118
Pisum sativum agglutinin	Reactive	d-mannose (l-fucose)	3	HCC, BLD	11, 125
Ricinus communis agglutinin 1	Reactive	d-galactose	3	HCC, YST	128
Viscum album agglutinin 1	Reactive	d-galactose	4		132
Peanut agglutinin	Nonreactive	d-galactose-1-3dgalNAc	NA	NA	122, 123
Phaseolus vulgaris erythroagglutin	Reactive	Complex biantenary glycan	5	HCC, YST, MHC	124, 125
Phaseolus vulgaris leucoagglutinin	Nonreactive	Complex tri- and tetranary glycan	NA	NA	125, 127
Wheat germ agglutinin	Nonreactive	GlcNAc (NeuAc)	NA	NA	120, 125
Soybean agglutinin	Nonreactive	d-galNAc	NA	NA	126
Griffonia simplicifolia 1	Nonreactive	d-galNAc	NA	NA	127
Dolichos biflorus agglutinin	Nonreactive	d-galNAc	NA	NA	123
Jackfruit agglutinin	Nonreactive	d-galNAc	NA	NA	11
Griffonia simplicifolia 2	Nonreactive	d-glcNAc	NA	NA	133
Ulex europeus agglutinin	Nonreactive	l-fucose	NA	NA	126

Note. PRG, pregnancy isoforms detected in amniotic fluid, umbilical cord serum, and maternal serum; HCC, primary hepatocellular carcinoma; MHC, metastatic (hepatoid) hepatocellular carcinoma found in extra hepatic tissues; YCT, yolk sac tumors; NA, nonapplicable to alpha-fetoprotein; LCH, liver cirrhosis; and BLC, benign liver disorder.

Phytohemagglutin-A (E-PHA), allomyrina dichotoma lectin (ALLO-A), and *Datura stromonium* agglutinin (DSA) has increased sensitivity and specificity of lectin reactivity with AFP (127). With the use of the new lectins and novel methodologies, the glycoforms of maternal serum AFP were found to be similar to those from umbilical cord serum AFP. This finding may be useful for evaluating the developmental state of the fetus by examining the nature of the AFP sugar chain. Initially, the use of MoAbs specific to AFP variants seemed to be unsuccessful. These early negative results may have occurred because the biantennary glycans of HAFP may be a self-antigen for mice, and because of the use of fetal calf serum, which may contain bovine AFP, with similar glycoforms as HAFP. However, recent studies using LCA-reactive and -nonreactive AFP demonstrated that LCA inhibited the binding of two MoAbs to HAFP, showing that a competition existed between the antibodies and LCA for the AFP sugar chain (12).

The present review was not intended to be all-inclusive for the carbohydrate microheterogeneity of AFP glycoforms. Therefore, the reader is referred to the extensive reviews on this topic published by Breborowicz, Lamerz, and Taketa (11, 126, 127, 337). Regarding the physiology of the microheterogenetic carbohydrate forms, rat AFP subjected to lectin columns shared higher estradiol binding affinities with less carbohydrate ($K_A = 10^{-8}$ M with 0.1–0.5 binding sites), while the high carbohydrate forms bound E_2 with lower affinity ($K_A = 10^{-7}$ M and 0.5 site) (135). Also, purified mouse AFP devoid of carbohydrate had very high-affinity binding sites for estradiol ($K_A = 0.7 \times 10^8$ M, $W = 3$) (136). Finally, studies showed that human peripheral lymphocytes were inhibited by AFP in the blast transformation assay in response to PHA and Con-A stimulation, but not pokeweed mitogen (137).

Isoforms of pH Heterogeneity. Isoforms of AFP have also been identified and characterized following exposure to changes in the pH environment. An early study by Alpert *et al.* (138) ascertained that HAFP could be separated into two major molecular forms by isoelectric focusing followed by classical chromatographic separation procedures. The two forms, found in both hepatoma and fetal sera, displayed isoelectric points of pH 4.8 and 5.2. The authors reported that a single homogeneous form (pH 5.2) could be produced after treatment with neuraminidase. Two subsequent reports followed in which another group of investigators demonstrated that the two isoelectric isotypes (pH 4.8 and 5.2) could be distinguished by their fatty acid content (139, 140). The pH 4.8 material contained 2.4 mol of fatty acids/mol of protein, while the pH 5.2 form contained no fatty acid. Removal of the fatty acids by treatment with charcoal converted all the AFP into material displaying an isoelectric point of pH 5.2. Addition of fatty acid to this HAFP solution again restored the protein to an isoelectric point of 4.8. More recent studies employing isochromatofocusing of HAFP in cord blood revealed the presence of three isoelectric variants (141). These pH isotypes included a pH 4.5 (52%); a pH 4.7 (43%); and an isovariant (at <pH 4.0) that could be eluted from the column by a 1.0 M NaCl solution. In this report, a pH 5.2 variant was not reported using cord blood as the AFP source. Conformational transition forms can also be induced in AFP by changes in pH as discussed later. AFP molecular transition forms have been reported at both acid and base extremes of the pH range (142). The conformational changes are reversible and gradual, indicating the presence of multiple transition forms of AFP. The authors of this latter paper reported that both rodent and HAFP had a remarkable hydrophilic exposed molecular surface at neutral pH and possess extensive hydrophobic segments hidden in molecular crevices as discussed in both the peptide fragment and epitope sections.

Antigenic Variants

AFP, as a member of the albuminoid gene family, displays 38% to 40% amino acid sequence identity to ALB (6, 117). Although unreactive in their native folded states, RIA utilizing reduced and carboxymethylated (CM) proteins as antigens reveal a serological cross-reactivity between AFP and ALB (143). Unfolding of the polypeptide chains by reduction of the disulfide bonds followed by CM produces derivatives that differ from the native state and crossreact in an RIA between AFP and ALB. It was further observed that ALBs from different animal species were equally crossreactive as HALB; overall, the AFP crossreactivity assays were found to lack species specificity (144). Although most serum proteins unrelated to AFP or ALB reacted little if any in the RIAs, transferrin (TRF) unexpectedly demonstrated a crossreactivity. No crossreactivity of TRF with AFP in its native states was observed; however, antisera to CM-AFP reacted with CM-TRF at high titers. Interestingly, the Gc DBP showed only moderate reactivity in this study. All the above RIA associations were further confirmed using Western blotting procedures. These results suggested that both TRF and Gc protein were structurally related to both AFP and ALB. Although Gc protein has been classified as an albuminoid family member, TRF is seemingly unrelated and these findings remain to be explained.

Immune Tolerance. AFP, when injected into a foreign species, produces a strong immune response, as does ALB. However, neither ALB nor AFP elicits an immune response in the species in which the proteins originated (145). Moreover, AFPs from different mammalian species are crossreactive and antibodies to HAFP induced in chickens recognized all mammalian species tested (146). Breakage of tolerance to autologous AFP can only be achieved using chemically modified or desialylated AFP injected with complete Freund's adjuvant into animals (147–149). Thus, the tolerance can be terminated by immunizing either with heterologous or hapten-modified homologous AFP, which results in production of antibodies that react with native host AFP (150). These antibodies crossreact with AFP in its native state, and the unfolded (reduced and CM) forms of AFP also show immunologic crossreactivity. The

antibodies produced in this fashion were found to diminish the AFP levels normally measurable in the host serum (151, 152). Such antibodies also diminish the elevation of serum AFP seen in hepatoma-bearing rodents, but do not protect against such tumors. In comparison, heterologous immunizations of mouse AFP into rats and vice versa have induced fetal death in pregnant animals (148, 153). The passive immunization of heterologous antibodies into hepatoma-bearing mice was found to induce pathological changes and suppress overall growth of these tumors (154). Furthermore, *in vitro* studies of such heterologous antibodies were found to be cytotoxic to murine hepatoma cells (155, 156).

Antigenic Determinant Sites. Serum ALB has been extensively used as an antigen. AFP is also a prime antigen when used as a foreign protein in a host species. The multiple antigenic determinant sites on proteins, lipoglycoproteins, and polysaccharides are termed epitopes, which are specific, limited parts of an antigen molecule that serve as inducers of antibody formation. When an antigen is employed to raise antibodies, the nature of the antigenic specificity should be considered for optimal usage. Induction of antibodies by an antigen molecule depends on rigidity of the molecule, presence of functional groups, size of the determinant sites, and the tertiary structure of the immunogen. Within an epitope, there is one area that is usually more dominant than the others even though the total determinant group may cover a large region. This area is known as the immunodominant site and often protrudes the farthest, but is not necessarily the terminal group (157). The immunodominant site comprises the major region of an epitope that confers individual and species variability and may even vary within an individual. In accordance with reported calculations and observations, the major epitopic site has a molecular size of about 10,000 Daltons; therefore, a molecule such as AFP (65 to 70 kD) should predictively display six to seven major epitopes (158). However, upon denaturation, as many as three internal minor sub-epitopes can be exposed per major epitope. The major epitopes are referred to as the functional valence of the molecule, while the internal sites exposed by denaturation are termed the nonfunctional valence (NFV). Hence, AFP should display at least six major epitopes (valence = 6) and possibly up to 12 additional minor epitopes (NFV = 12) for a possible total of 18 antigenic sites.

Epitope Analysis. A summary of the epitope analysis of HAFP with an array of MoAbs was reported at an international workshop in 1998 (159). Previous reports using polyclonal antibodies had inferred that HAFP indeed displayed at least six major epitopes (160). A summary of the findings of the 1998 workshop was published in a series of four consecutive reports. Using 30 different antibody clones against HAFP, two reports confirmed the presence of five to six major epitope clusters and identified several additional minor epitopes (161, 14). One of the major epitopes did apparently contain carbohydrate as part of the immunodominant site specificity as reported in these studies (12). Another of the epitopes identified in the HAFP molecule demonstrated both open and cryptic (hidden) forms (13). An overall survey of the literature including both mono- and polyclonal antibodies to AFP was in agreement that four to eight distinct epitopes are present on the AFP molecule (162–171) (Table VI).

Epitope Mapping. A study described at the international workshop by Norwegian investigators reported six major epitopes and an additional seven sites of low antibody-binding activity, suggestive of minor epitopic sites (161). The six major epitopes were labeled A, B, C, D, E, and X (a partially hidden site) and were represented by antibodies of high binding affinity (Fig. 2C). A second group led by Russian and Dutch scientists (14) reported at least 11 antigenic clusters, which were categorized into six distinct major epitopes (A–F) derived from 30 HAFP MoAbs. These MoAbs to HAFP exhibited four means by which cross-reactivity occurred: full competition; partial competition; full independence, and enhancement of binding. Additional complexity was encountered when some MoAbs reacted with AFP in solution while others reacted only with AFP adsorbed to a solid surface. Thus, some epitopes were hidden or cryptic in nature and were expressed only on partially denatured AFP adsorbed to plastic or nitrocellulose membranes (NCM). Finally, it was observed that the MoAbs reacted similarly with the major epitopes whether HAFP was derived from umbilical cord serum, placental extracts, or tumor tissue. However, minor epitopic differences between cord sera and placental extracts were found, suggesting conformational alterations in AFP forms from the different tissues (14).

Studies presented by Russian investigators at the workshop (see above) found additional MoAbs reacting against subfractions of native HAFP, suggesting the presence of at least four additional minor epitopic variants (13). These subfractions of AFP displayed similar molecular weights to the native AFP molecule, but showed different serological reactivity when conformationally altered by fixation on NCMs. These epitopes existed in two forms on the native AFP molecule: an open and a cryptic form. Since the hidden sites could be revealed after partial denaturation, these subfractions represented conformational variants that lie partially buried in the compactly folded, intact molecule. Recently, cryptic epitopes on HAFP have been reported to induce spontaneous immune responses in human patients suffering with hepatomas, cirrhosis, and chronic hepatitis (172). The physiological significance of hidden subdomain epitopes on HAFP has emerged as a fertile field for both basic and clinical investigations of AFP-regulated ontogenic and oncogenic growth.

One such hidden epitope on the AFP molecule (P149 peptide) did not react in commercial radioimmunologic, immunoenzymatic, or chemiluminescent assays designed to measure HAFP levels in pregnant and tumor-bearing patients. In contrast, polyclonal antibodies produced in rabbits using keyhole limpet hemocyanin conjugates produced a

Table VI. The Major and Minor Epitopes on Human Alpha-Fetoprotein Are Categorized in Accordance With Immunoglobulin-(Ig) Subclass, Number of Cell Clones, and Scientific Utility

Group	Hybridoma clones	Major epitopes	Minor[a] epitopes	Ig class	Scientific utility	Reference
Ruoshahti (1980)	22	4	NS	IgG (kappa)	Serum, amniotic fluid RIAs	162
Mayumi (1982)	11	1 determinant major group	1 determinant minor group	NS	Reduced and alkylated forms; ascites fluid and cord sera AFP	163
Brock (1982)	12	(5 total) 3 2	NS	IgG, IgA	Pregnancy sera RIAs	164
Micheel (1983)	7	4	NS	IgG_1 IgG_{2a} IgG_{2b}	Solid phase immunofluorescence MoAbs	165
Kaplan (1984)	6	4	NS	IgG	Isoelectric focusing isoforms variants	166
Moreno (1988)	8	6–7 (Group 1)	1 (Group 2) 4 (Group 3)	IgG_1 IgG_2	Cord blood isoform variants	167
Alpert (1989)	Polyclonal antisera	6	NS	Total IgG	Experimental epitope calculation	159
Yazova (1990)	12	6	6	IgG_{2a} IgG_{2b} IgG_1	Present in normal and malignant condition	168
Novo (1990)	5	NS (5?)	NS	IgG_1	Cord serum analysis hydrophobic affinity	169
Chakraborty (1991)	14	I, II, III, IV, V	NS	IgG_1, IgG_{2a} IgG_{2b}, IgA IgM	Cord serum analysis, avidity measurement	170
Christiansen (1994)	24	6 (A, B, C, D, E, F)	NS	IgG_1 IgG_{2a} IgG_{2b}	HAFP epitope mapping	160
Abelev (1994)	Mo Abs and poly Abs	4	4 (subfraction analysis)	IgG	Immuno-affinity on Porous membranes	171
Nustad (1998); ISOBM workshop	30	6 (A, B, C, D, E, X) (high affinity)	9 –2 low binding, –7 very low binding	IgG (Non-specific)	RIAs from pools of cord and tumor sera	161
Yakimenko (1998); ISOBM Workshop	28	6 (including one cryptic site)	4 (non-native; low binding)	IgG_1 IgG_{2a} IgG_{2b}	Studies of epitope mapping from cord and placental AFP	14
KARMORA (1998); ISOBM Workshop	30	6	4	IgG	Study of conformational variants, denaturated forms (molecular weight)	13
Taketa (1998): ISOBM workshop	30	2 (LCA and con-A associated)	0	IgG	MoAb studies of lectin interaction	12

All antibodies demonstrated binding affinities between 10^{-6} M to 10^{-9} M. Note the increase in major detection of epitopes with chronological time. NS, Not stated; Ig, Immunoglobulin; LCA, Lens culinaris agglutinin; ConA, Concanavalin-A; MoAb, Monoclonal Antibodies; and ISOBM, International Society of Oncodevelopment Biology and Medicine.
[a] Conformational variants

moderately high-titered P149-peptide antiserum (1:10,000), which was subsequently adapted to direct and indirect ELISA employing an alkaline phosphatase labeled antibody. By means of the peptide-ELISA it was demonstrated that the antibodies could not detect the amino acid peptide sequence on intact, native HAFP that had been purified from term cord serum (173). However, the immunoreactivity of the peptide antiserum was demonstrable following treatment of native HAFP with high concentrations of estrogen or by solid-phase adsorption of HAFP to the microtiter plate surface. The antiserum also was capable of detecting the peptide in amniotic fluid during pregnancy and in the serum of patients with AFP-secreting tumors.

In summary, knowledge of the antigenic structure of AFP is both important and necessary for several reasons. First, knowledge of the major and minor epitopes on HAFP could lead to more rational designs of monoclonal/polyclonal antibodies for immunoassay kits produced by the various pharmaceutical companies. Such designs might include both the major epitopes on the native AFP molecule and the minor (hidden and exposed) epitopes observed in the conformational variants of AFP. It may also be important to know the ratio of conformationally transformed to native AFP in the serum to determine whether it may relate to disease states and/or cancer stages. Such information might prompt further comparative studies among the various kit manufacturers, as well as analysis of specific functional groups on the AFP molecule itself. Such information might also be employed to identify AFP domains or motifs responsible for some of its functions such as ligand binding/transport, cell receptor interactions, growth regulation, and immunoregulatory activities. Finally, these findings could

aid in characterizing and distinguishing the variant states of the AFP molecule such as the compact native form, denatured intermediates, conformational variants, and the MGF (see next section).

Denatured Intermediates and the MGF

Proteins that reside in living cells are thought to assume a conformational state unlike that of the native, secreted molecules found circulating and/or stored in biological fluids (15). The highly variable environments of the different intracellular compartments dictate that a protein exist in a mildly denatured (unfolded) state, which has been termed the MGF (15). As discussed below, the MGF has now been implicated in a variety of physiological activities including protein translocation, insertion into membranes, heat shock protein (HSP; chaperone) binding and recognition, and protein degradation by lysosomes and/or the ubiquitin system. First discovered in 1981 (174), the MGF represents an intermediate folded state of a protein induced by mild denaturing conditions in contrast to harsh exposure to strong ionic solutions, detergents, and extreme pH values, which cause aggregation. This slightly unfolded state of a protein has now been demonstrated for many different naturally occurring proteins including HAFP (175–196) (see Table VII). Although the MGF can be induced *in vitro* by low pH, moderate ionic strength solutions (guanidinium chloride [GnCl], urea, etc.) and high temperatures, the MGF can also be produced in physiological environments by point mutations, removal of ligands, and disruption of disulfide bonds (15, 176, 177). The MGF can readily be studied using low and high pH or moderate organic solution concentrations with techniques such as near and far ultraviolet circular dichroism, infrared spectra, and nuclear magnetic resonance (NMR). Extremes of the conditions stated above produce a completely unfolded state, which is the totally denatured or aggregated form (Fig. 4 and Table VII). However, it must be noted that not all proteins have been shown to form stable molten globule states or to fold through MG intermediates.

Characteristics of the MGF. The MGF is thought to represent a third thermodynamic state of protein molecules induced by mild denaturation (15, 197) (Fig. 4). Formation of the MGF proteins are proposed to occur in a four step sequence as follows: the native-like state; a transitory unfolded form; the true MGF; and the denatured form (see Fig. 4). The MGF, once considered to be an equilibrium state at mild denaturing conditions, is now viewed as a kinetic intermediate in the protein folding process (197). Thus, the properties of the MGF are its relaxed compact-

Table VII. Alpha-Fetoprotein and Other Proteins That Form Molten Globule Intermediate States Are Listed (see Fig. 4)

Protein name	Molecular weight (KDa)	Denaturing agents	Assay methods	References
Human alpha-fetoprotein	70	Acid pH, urea, ligand removal, temperature	Far to near CD, trp Fluor, scan MCA viscom	50, 175–178
Human serum albumin	65	Temperature, urea, ligand removal, ionic changes	CD, Fluor, spectroscopy scanning micrcalorimetry	176, 186
Alpha-lactalbumin	14	Acid pH, ionic changes, ligand removal	Differential scanning microcalorimetry, CD	179–182
Beta-lactoglobulin	18	Alcohols, DMFA, dioxane	Far to near CD, Trp Fluor, Fluor decay	183
Type C lysozyme	14	Temperature, amino acid mutation	Glycosidase enzyme activity	184
Transthyretin	15	Acid pH, amino acid mutation	Far to near CD, Fluor, SANC, SDS-PAGE	187
Bovine serum fetuin	48	Temperature, GNHCl	Differential scanning, Microcalorimetry trp Fluor, GF	185
Retinol-binding protein	21	Acid pH	Far to near CD, trp Fluor, NMR viscosity	188
Cytochrome-C	13	Acid pH, alcohol	Far to near CD, trp Fluor, MCAL, diffusion	189
FK506-binding protein	12	Urea, aqueous buffers	Far to near CD, NMR	190
Dihydrofolate reductase	17	Temperature, urea	Stopped-flow Fluor, ligand binding	191
Carbonic anhydrase	30	GNHCl, pH, temperature	CD, Fluor, ANS binding	193, 194
Steroid reg-protein	37	pH, urea, temperature	CD, Fluor, proteolysis	192
Colcin-A	50	Temperature, pH, acid, site-directed mutations	Microcalorimetry, CD, ion channels	195, 196

Note. Fluor, fluorescence; NMR, nuclear magnetic resonance; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; Trp, tryptophan; GNHCl, guanidine hydrochloride; Mut, mutagenesis; SANC, small angle neutron scattering; MCAL, microcalorimetry; and ANS, fluorescent hydrophobic dye.

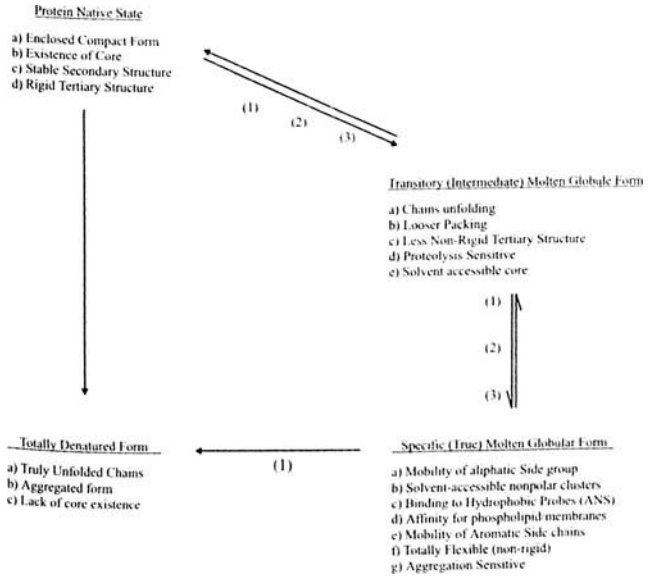

Figure 4. Flow diagram showing the properties and progression of a native protein (see Table 7) to the MGF derived from *in vitro* studies (Refs. 178–186). Directional arrows indicate bi- and unidirectional flow reactions. The numbered parentheses signify predicted multiple intermediate steps in the progression toward the MGF.

ness, stable secondary structure, nonrigid tertiary structure, and ability to bind hydrophobic probes due to its less rigid tertiary structure.

It is surprising that the MGF, rather than the classical native protein, has been proposed as the common form within the cell for various physiological purposes (15, 198). The cytoplasm of the living cell is teeming with high concentrations of proteins, nucleic acids, and organelles at low pH (4.5–5.0), which commonly encounter heat and osmotic shock, variable electrostatic fields, and a myriad of membrane interfaces (199). Although intracellular environments might not represent the extreme denaturation conditions that can be produced *in vitro* (urea, GnCl), cytoplasmic proteins do endure mild denaturation states, which probably alter their overall molecular structure (198, 199). In this more flexible form, a protein can readily adapt itself to changing cell conditions such as interaction with chaperones, membranes, stress/shock extremes, and encounters with other intercellular components and organelles. As hypothesized in Fig. 4, AFP (as example protein) in its intact, native form would embody a compact, rigid molecule with an inaccessible central core (Fig. 5). When exposed to a stress/shock microenvironment (i.e., high serum estrogen levels from the mother), the fetal AFP molecule might progress through one or more intermediate forms (representing molecules exposed to slightly increased increments of denaturation) to arrive at a transitory MGF with slightly looser packing and unfolding chains (Fig. 4, top right). This transitory (intermediate) MGF of AFP would be further characterized by a slightly less rigid tertiary form with a central core accessible to solvents. This transitory AFP form, upon continued exposure to the stress/shock state, might then pass through one or more stages toward the true MGF of AFP. The specific (true) MGF of AFP represents a totally flexible molecule with solvent accessible nonpolar residues and complete mobility of both aromatic and aliphatic side chains. This flexible form of AFP is more protease sensitive, binds hydrophobic ligands (dyes), and displays affinity for cell membranes (Fig. 6). Up to this point, both the transitory and true MGFs are in dynamic equilibria (two-way arrows, Fig. 4) depending on their molar ratios; however, the true MGF is aggregation sensitive and might eventually be susceptible to irreversible denaturation (Fig. 4, lower left). This hypothetical form (MG) of HAFP might comprise only 1.0% or less of the total circulating fetal protein (247), but might be expected to increase in the presence of fetal defects (173) and be the estrogen-binding form of HAFP previously described (339, 340).

AFP in the MGF. Laboratory studies have shown that the conformation of AFP is modified following changes in the microenvironment, i.e., increased serum concentrations of hydrophobic ligands such as steroidal estrogens and fatty acids (176–178, 8). It has been reported that HAFP, derived from cord serum, can be induced to undergo a conformational change to the MGF (175). HAFP in this type of configuration would lack a rigid tertiary structure, but would have a native-like content of secondary structure and

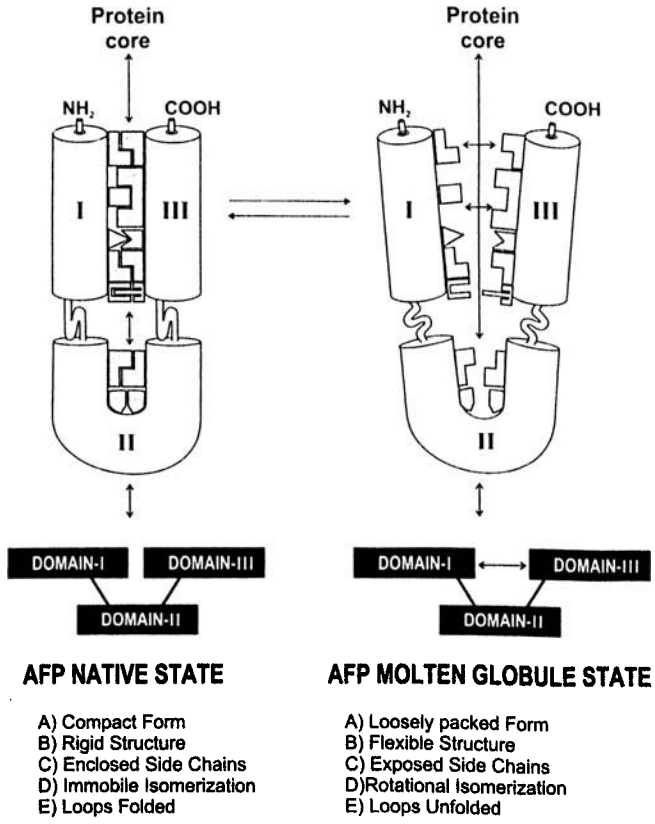

Figure 5. Diagrammatic representation of the proposed tri-domain structure of HAFP transitioning from the native to the MG state. This artist's conception is predicted from data derived from Refs. 15, 189, 175 through 178, 194, 197, and 203.

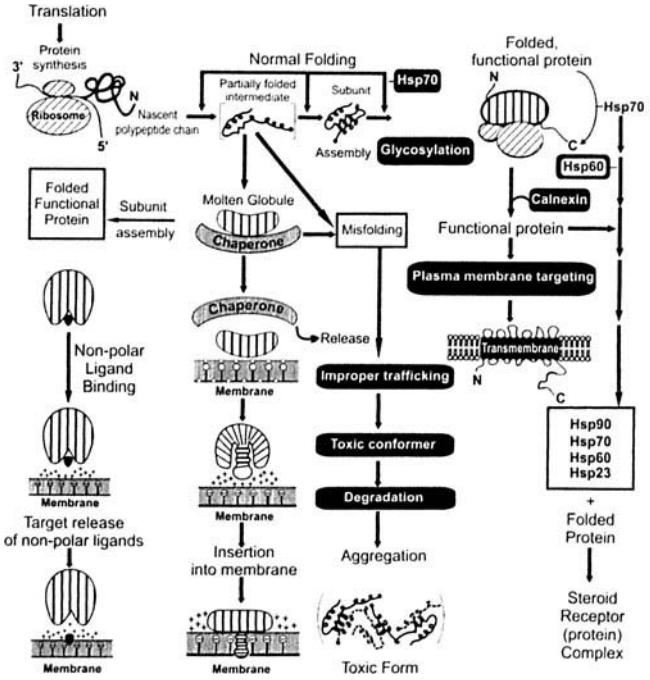

Figure 6. Flow diagram depicting the pathway of the molecular chaperoned folding of ribosome-translated nascent polypeptide chains through the normal folding process to the functional protein. As shown, the partially folded intermediates can be passaged through a MG stage or deemed a misfolded protein destined for degradation. The far right side pathway depicts a folded protein that can assume a variety of functions. The far left side pathway displays a folded protein transporting a bound ligand to a membrane surface, while the near left side pathway depicts insertion and passage of the MG intermediate form through a membrane interface. Concepts for this composite diagram were taken from Refs. 15, 195, 196, 198, 189, 207, and 208.

would be intermediate between the native compact form and the completely denatured form (see Figs. 4 and 5). Such denaturing conditions might include anoxia, pH and osmotic extremes, glucose and heat shock, ischemia, and excessive ligand (i.e., estrogens) concentrations. Ligand-induced events are known to induce conformational changes in the HAFP molecule *in vitro* that expose a site on the protein, which inhibits the growth of estrogen-sensitive cells in a cell regulatory fashion (4, 7, 87, 115). Like other hidden (occult) epitopes on AFP, other sites can be uncovered by fatty acid exposure and solid surface adsorption. While ALB reverts immediately back to the native state following shock or stress at equimolar ratios, AFP was found to retain the mildly denatured intermediate configuration in an irreversible form, leaving any exposed peptide segments intact (176). However, a subsequent report showed that increased ligand molar ratios could revert MG-AFP back to a more native form (178).

The MG configuration for AFP showed a flexible tertiary structure with a native-like content of secondary structure; therefore, HAFP in this form constitutes an intermediate stage between the native compact form and the completely denatured (unfolded or aggregated) form (175, 177, 200). AFP in the MGF would be quite flexible, exhibiting intrinsic rotational properties; its loosely packed surfaces would expose hydrophobic, aliphatic, and aromatic sidechains that are normally concealed in the native state (Fig. 5). Proteins are thought to assume the MGF as they approach a charged surface or a membrane (196, 201). Once across the membrane itself, the protein refolds to the native state. Calculations have shown that the dielectric constant of water (and buffers) near the flat nonpolar organic surface is one-half of its intrinsic dielectric constant (183, 189). Therefore, a decrease of the water dielectric constant near a membrane can facilitate a local decrease in pH sufficient to transform native proteins into the MG intermediate state. It is the electrostatic attraction of positively charged groups of a protein with the negatively charged membrane surface that brings the protein and the membrane into juxtaposition (189). It has, therefore, been proposed that the MGF is involved in protein adherence, translocation, and insertion into membrane bilipid surfaces (15) (Fig. 6). Indeed, the use of MoAbs has demonstrated that HAFP purified from placental extracts displayed different epitopes compared with AFP passaged into the umbilical cord serum (14).

Induction of the MGF. The AFP molecule at pH 3.1 undergoes a considerable conformational rearrangement and fulfills all the requirements of the MGF (175–177). The acid form of HAFP preserves a native-like content of secondary structure, but has a less compact tertiary structure than the native protein molecule. The affinity of AFP for the hydrophobic fluorescent probe, anilino-napthalinesulfate (ANS), is much higher than that observed for the completely folded native state (175). It has been shown that ANS binds to solvent-accessible clusters of nonpolar groups in the native state (194) and that this binding is even stronger in the MGF than in native AFP. This transformation of native AFP to the MGF appears to result in a substantial increase in flexibility, which facilitates the release of hydrophobic ligands that were bound to the native form (177). Thus, the MGF of HAFP may be of physiological and or pathological significance as discussed below.

The release of hydrophobic ligands from HAFP results in a conformational rearrangement of the molecule to a less compact form, defining the MGF (177). It has further been reported that stripping off the E_2 ligand of HAFP is an irreversible process (at equimolar ratios of E_2 and AFP). It is conceivable that exposing high concentrations of estrogens to HAFP can also produce this state (115). It has been proposed that the interaction of ligand-saturated AFP with cell surface receptors induces a conformational change in the protein accompanied by dissociation of the AFP-ligand complex for delivery to the cell (202) (Fig. 6). The ligands are then translocated to nearby ligand-acceptor sites on the cell surface. The ligand-free AFP molecule can then dissociate from the AFP receptor and be transferred into the cell by endocytosis (see 201, 202).

The denaturation of AFP by an increase in temperature or by exposure to high urea concentrations is irreversible and also results in ligand release from the AFP molecule

Protein	Amino Acid* Sequence #	Amino Acid Sequences	Percent (%) Identity/Similarity	Percent (%) Total
Human AFP	445-480	L S E D K L L A C G E G A A D I I I G H L C I R H E M T P V N P G V G	100/0	(100)
Rattine HSP70	1335-1347	I F F S P L L D C G E G	50/15	(65)
Pisum s. HSP70	552-558	A X E E G X S Q E C I G K L C I Q H E	50/28	(78)
Petunia HSP70	533-550	A E E E G G S X E C I G E L C L Q H E	50/22	(72)
Clover HSP70	22-39	A Q E E G X S K E L I G E L C L Q H E	50/14	(64)
Tobacco HSP70	455-471	V D E E K S G X E L I G E L C L Q H E	50/14	(64)
Yeast CALMD	77300-77311	E L V N R I G Q L C I R L K	44/0	(44)
Human CALRCT	1535-1547	I Q S I I V G H L G W F Q X	38/15	(53)
Murine PDSI	130-131	T A D G I V S H L K K Q A G	36/21	(57)
Human CALC	1535-1547	R K F G S T L L I C I R H S	28/14	(42)
O. Sativa UDGT	335-353	N K K G K L M X M T P P S P G M T	42/35	(77)

* = Genebank derived
Pea = Pisum sativum
Petunia = Petunia hybrida
Clover = Trifolium repens
Tobacco = Nicotiana tabacum
O. Sativa = Oryza sativa
HSP70 = Heat Shock Protein BiP70 family
CALMD = calmodulin
CALRCT = calrecticulin; ss-A/RO
PDSI = protein disulfide isomerase
CALC = calcitonin
UDGT = UDP-glucose glycosyltransferase
X = unknown amino acid

Figure 7. Matching of amino acid region #445–480 of human alpha-fetoprotein (AFP) with conserved amino acid sequences of heat shock protein (HSP) related factors.

(175). After ligand release, the AFP molecule displays all the properties of the MGF (15). This suggests that the rigid tertiary structure of AFP is controlled by interaction with ligands, while their release results in the MGF (177). Unlike AFP, human ALB denaturation and unfolding appear to be more readily reversible, but may be dependent on ligand: AFP molar ratios. The release of ligands from ALB resulted in only small decreases in stability, but ALB did not undergo extensive MG formation. The authors of this report suggested that glycosylation sites of the AFP molecule may be responsible for the lack of reversibility since ALB contains no such N-glycan sites (176).

AFP appears to be largely a growth promoter rather than an inhibitor, as revealed by a detailed literature survey and the observation that the majority of human pregnancies (>95%) are successfully brought to term without intrauterine growth retardation. However, a small percentage of post-translationally modified AFP molecules have been reported to possess growth inhibitory properties in both *in vivo* and *in vitro* assays (173, 309). This small percent of AFP molecules, present in both naturally occurring and cell culture forms, can be increased *in vitro* by mild denaturation methods such as exposure to solutions of high ionic strength and excessive ligand concentrations (50, 310). Intracellular and intercellular environments such stress/shock-induced conditions might favor conformational changes in the protein, which permit expression of transitory intermediate forms of an energy state that would be consistent with the MGF (15). Similar molecular folding changes have been reported to occur in the ER and are associated with the HSP chaperones (see references below). It has been proposed that altered conformations of the AFP molecule expose cryptic sites (amino acid stretches or motifs) that are normally concealed in molecular clefts in the native form (87, 172). Some of these hidden sites have since been reported, as discussed previously.

Role of Molecular Chaperones

The MGF has a slightly altered tertiary architecture, a property needed to adapt proteins to different cell conditions. The presence of solvent-accessible, nonpolar clusters on the surface of the MGF may serve to facilitate interactions with HSPs and related chaperonins (203, 204). There is now direct evidence that chaperonins only function is to mediate the correct assembly of nascent polypeptides (205). Thus, the chaperonins keep nascent proteins in the MGF to prevent folding at improper times and inappropriate cell locations. The chaperonins function not only during periods of stress (i.e., heat shock, poisoning, ischemia, anoxia, and osmolality), but also during normal cell functions such as nuclear receptor complexing, storage, and cell trafficking (see Fig. 6).

Chaperonin include not only the HSP and glucose shock proteins (HSP90, HSP70, HSP60, and BiP), but also the small HSPs, co-chaperonins, calcium-related proteins (calnexin and calreticulin), and two enzymes, namely, protein disulfide isomerase and peptidyl-prolyl isomerase (205, 206) (see Fig. 7). The chaperonins are not enzymes and do not accelerate protein folding, but rather they ensure that proteins fold correctly and in the proper sequence. They interact with newly synthesized polypeptides and prevent nonspecific aggregation and keep proteins as inactive monomers, thereby facilitating specific assembly; they also assist proteins in survival and repair following heat and/or other stresses (207, 208). Thus, chaperonins prevent nonspecific aggregation following protein release from ribosomes and promote renaturation after temperature shock. Since MGs usually have a tendency to aggregate, it is the function of chaperonins to prevent such clumping (208, 209). In fact, it is believed that chaperonins bind to proteins in order to keep them in their MG state; thereby chaperonins retard rather than accelerate protein folding (181, 209). It is

germane to this discussion that a hidden epitope on HAFP (amino acids 445–480) was initially discovered by amino acid sequence matching to HSP-70 and its cognate proteins (87, 173). In the matching, HAFP sequences amino acids 452 to 458, 460 to 466, and 475 to 480 were found to display identity/similarities to eukaryotic HSP-70 sequences (Fig. 7). HSP-70s are known to interact with incompletely assembled proteins in which only a limited internal segment of polypeptide chain is exposed (210). This stretch of HAFP amino acids was later shown to emerge following incubation in high estrogen concentrations (173). This segment may represent one of several sites on HAFP to which HSPs bind during the assembly of the HAFP molecule following release from the ribosome. HSP70 proteins are known to bind to short, extended, and exposed hydrophobic peptide sequences of larger proteins (210, 211).

Misfolded Proteins and Genetic Disease. The ability of a polypeptide to fold into a unique, functional, three-dimensional structure is determined by its amino acid sequence, the chaperonins, and the enzymes that catalyse the folding (see 205). HSP70 binds to unstructured regions of newly synthesized polypeptides through exposed hydrophobic residues (Fig. 7). The masking of these surfaces prevents aggregation of the partially folded chains, which can prove toxic to the cells as reported for Alzheimer's disease (AZD) and the amyloid disorders (2). The HSP70 bound-polypeptide is then transferred to the HSP60 chaperonins, which aid in transport into organelles and continued assembly into oligomeric complexes with the smaller HSPs (205, 206). In mammals, HSP70 and HSP60 are found in mitochondria, cytosol, and ER, whereas the HSP70 homologue BiP (immunoglobulin-binding protein) is found only in the lumen of the ER (205). The HSP70 is associated with clathrin-coated vesicles and is cytoplasm retained, while BiP is confined to the ER (210). Evidence is accumulating that defective or altered protein folding (mutations and/or modifications) may form the basis of many human diseases (212–214, 325).

As evidenced in Table VIII, improper folding, assembly, and localization of nascent proteins cause them to aggregate and deposit abnormally in and around cells (215–238, 318–324); misfolding of proteins can lead to debilitating diseases and disorders and sometimes death (213). It is tempting to speculate that congenital disorders involving altered concentrations of HAFP (Down's syndrome, chromosomal trisomies) may be associated with defective protein folding/assembly and the involvement of conformational variants. Down's syndrome is characterized by low HAFP levels in both amniotic fluid and maternal blood, which could be indicative of either low hepatic AFP synthesis or a conformational alteration of the secreted protein that eludes serological detection (215–219). Improper folding/assembly can occur at two possible checkpoints: on nascent polypeptide chains as they emerge from the ribosome during translation or following passage through a membrane during translocation (Fig. 7). Since a normal mRNA transcript has been detected in the livers of prenatally detected Down's syndrome babies, a defect in hepatic protein synthesis appears less likely (215). It remains plausible that such fetal defects might be related to misfolding/misassembly of the fetal protein following either synthesis in the hepatic cell or during passage from the fetal to the maternal circulation via the placental membranes. To date, the data from Down's syndrome pregnancies seem to favor a conformational alteration of AFP resulting from impaired fetal kidney processing (pyelectasis) and/or altered transmembrane and transplacental passage into the maternal circulation (218, 240, 241).

AFP Intermediates and Genetic Disease. Down's syndrome is similar to AZD in that aggregated pro-amyloid and amyloid fibrils gradually accumulate in brain cells (242). In AZD, an increased synthesis and accumulation of HSP70 proteins have been reported (243) and it has been proposed that the aetiology of AZD may involve a misfolded chaperone protein (244). The gene for the pro-amyloid precursor is on the long arm of chromosome-21, which is present in triplicate in Down's syndrome. Overexpression of this gene is thought to lead to increased levels of pro-amyloid/amyloid deposition in Down's syndrome leading to early onset AZD. Abnormal accumulation of proteins in the ER serves as a eukaryotic stress signal and triggers the activation of heat shock genes (245). This unfolded protein response, induced by the accumulation of proteins in the lumen of the ER, results in upregulation of genes encoding ER-resident enzymes involved in the protein folding process (246). A thermokinetic model has been employed to calculate that, in any population of proteins, a small portion (1% or less) might be randomly misfolded (247). Misfolding would most likely result in the exposure of parts of the protein chain that are normally hidden inside the molecule, areas to which molecular chaperones bind (Fig. 7) (325). Normally, these parts become buried as the protein folds and the chaperone is then released (173). Usually, the only consequence of improper folding would be a loss of biological activity in the protein that eventually aggregates. The misfolded protein would either be refolded by another chaperonin or eliminated from the cell; however, slightly misfolded forms can exocytose the ER (336). However, when the misfolded protein is a chaperonin that modulates the folding of other proteins, the outcome could be significant. If Down's syndrome is a disease of a malfunctioning or misfolded chaperonin, as postulated for AZD (244), one could speculate that an increased proportion of other misfolded proteins (i.e., AFP) might be expected. It is noteworthy that both Down's syndrome (215) and insulin-dependent diabetic pregnancies (248) exhibit a 20% decrease in serum and amniotic fluid AFP levels. One could propose that misfolded and/or MG intermediates of secreted AFP might be nonimmunoreactive and hence undetectable by conventional immunoassays, examples of which are known to exist (44–46).

Table VIII. Selected Proteins and Their Proposed[a] and/or Confirmed Folding Diseases and Disorders

Protein involved	Disease/disorder	Protein defect	Molecular manifestation	Reference
Alpha-fetoprotein	Down syndrome, chromosomal abnormalities	Inability to fold	Misfolding, altered HSP70 interaction	215–219
Serum albumin	Chronic liver[a] disease	Improper folding	Misassembly, aggregation	220–222
CF-TMCR	Cystic fibrosis	Misfolding	Altered HSP70-calnexin interaction	223, 226
Fibrillin	Marfan syndrome	Inability to fold	Misfolding	237
α-Ketoacid dehydrogenase complex	Maple sugar urine disease	Misfolding	Misassembly	235, 236
Prion protein	Scrapie, Creutzfeldt Jacob disease	Toxic folds	Aggregation	230
Rhodopsin	Retinitis pigmentosa	Mislocalization	Improper trafficking	228
Superoxidedismutase	Lou Gehrig's disease	Inability to fold	Misfolding	212
P53 Protein	Cancer	Misfolding	Altered HSP70 interaction	239
β-Amyloid	Alzheimer's disease	Toxic folds	Aggregation	231
β-Hexosaminidase	Tay-Sachs disease	Mislocalization	Improper trafficking	234
Type-1-α procollagen	Osteogenesis Imperfecta	Misassembly	Altered BiP interaction	227
Transthyretin and isozyme	Familial amyloidosis	Toxic folds	Aggregation	229
α_1-Antitrypsin	α_1-Antitrypsin deficiency	Mislocalization	Improper trafficking	224
Collagen	Scurvy	Inability to fold	Misfolding altered HSPs	232
Crystallins	Cataracts	Toxic folds	Aggregation cross-linking	193
LDL receptor	Familial hypercholesterolemia	Mislocalization	Improper trafficking	233
Insulin receptor	Leprechaunism	Mislocalization	Improper trafficking	223
Tau protein	Alzheimer's disease	Abnormal Phosphorylation	Aggregation, proteolysis	238
Hemoglobin	Sickle cell anemia, β-thalassemia	Toxic folds	Aggregation, polymerization	212
Acyl-CoA dehydrogenase	Hereditary MCAD deficiency	Misfolding	Impaired protein assembly	225
Thyroglobulin	Congenital goitrous hypothyroidism	Defect in folding	Misassembly and ER retention	318
Unknown protein	Carbohydrate-deficient glycoprotein syndrome	Insufficient glycosylation	Retention in the ER	319
Vasopressin receptor	Nephrogenic diabetes insipidus	Misfolding	Retention in the ER	320
GPIIb integrin	Glanzmann's thrombasthenis	Improper folding	Retention in the ER, aggregation	321
Von Willibrand factor	Von Willibrand disease	Misfolding	Impaired transport from ER	322
Clotting factor VII	Hereditary factor VII deficiency	Misfolding	BiP retention in ER	323
Protein-C	Protein-C deficiency	Improper folding	GRp-94, BiP retention in ER	324

MCAD = Medium chain acyl-CoA dehydrogenase; LDL = Low density lipoprotein; CF-TMCR = Cystic fibrosis transmembrane conductance regulator; Proposed Folding Disease; BiP = glucose induced shock protein, immunoglobulin binding protein

There is now direct evidence that some proteins unfold to MG intermediates for import and translocation across cell membranes (196, 201) (Fig. 6). Observations ascribed to this translocation phenomenon have also been associated with HAFP passage through the umbilical cord and across the placenta. MoAbs have been utilized to enumerate the major and minor epitopes on cord serum-derived versus placental-purified HAFP. Epitope mapping of HAFP from cord serum versus placenta extracts has revealed an increase in the number of minor (hidden or occult) epitopes detected on placental AFP (14). Recent studies further demonstrated that HAFP at the placental interface is exposed to high concentrations of fatty acids and other hydrophobic ligands (8, 249), which are known to induce conformational changes in the AFP molecule. These data would suggest that HAFP might assume the mildly denatured transitory (intermediate) state of the MGF when traversing the placenta (see Fig. 4). In light of these recent findings, the transplacental passage of AFP will be addressed in more detail in the following section.

Update on AFP Physiology and Clinical Potential

Transplacental Passage of AFP. The fetal-to-maternal transfer of AFP occurs by a transplacental, not a solely transamniotic, route as previously postulated (250). The transfer of AFP across the placenta, once thought to be accomplished only by paracellular diffusion (251), involves additional and more complicated mechanisms (Table IX). In

Table IX. Summary of Various Biological Activities of Human Alpha-Fetoprotein Regarding Physiological Mechanisms and Experimental/Clinical Significance

Biological activity	Ligand or binding proteins involved	Implicated physiological mechanism	Experimental or clinical significance	Reference
A. Placental transfer/ passage	Fetal-AFP; Amniotic fluid-AFP; Cord-AFP	Paracellular diffusion; unidirectional passage; hydrostatic gradient	Placental defects; abnormal MSAFP levels	250–256
B. Drug conjugation, induced cytotoxicity	Doxirubricin, daunamycin, calichemicin, bleomycetin methotrexate	Receptor-mediated endocytosis; formation of endosomes, receptosomes	Multi-drug resistance; anti-cancer therapeutics	260–263
C. Ligand binding and carrier function	Dioxin; bioFlavonoids; phytoestrogens	Hydrophobic binding pockets	Fetotoxins, carcinotoxins endocrine disrupters	264–268, 292
D. Control of growth; up- and down-regulation	a. caspase-3 TNFR TNF b. Transcription factors c. Arrestin, dynamin	a. Apoptosis b. Cytoplasmic signalling c. Agonist desensitization	a. Caspase activation b. Mitogenesis down-regulation c. Receptor blockade, signal uncoupling	282, 284, 289, 310 297–300 275, 302–309

Note. TNF, tumor necrosis factor and TNFR, tumor necrosis factor receptor.

an elegant study utilizing perfused normal human placental cotyledons, the mechanism of transplacental transfer of AFP was further elucidated (253). Four anatomical barriers must be traversed between the maternal and fetal circulation in the human. These transplacental barriers include the syncytiotrophoblast bathed by maternal blood in the intervillous space, the trophoblastic basement membrane, the capillary basement membrane, and the fetal capillary endothelium. The transplacental passage of AFP was found to be asymmetrical and unidirectional, displaying a faster transfer rate of AFP from the fetal to maternal circulation than vice versa. Fetal AFP was found to enter the maternal circulation via two possible pathways (252, 253). The first pathway (containing two parts) involved AFP exiting fetal vessels and passing through the placental villous core; AFP can also traverse fibrinoid deposits and cross at sites of discontinuity of the syncytiotrophoblast cells. Thus, AFP can enter the maternal circulation with and without passage through the cytoplasm of these cells. The second pathway involves AFP gaining entrance into the decidua basalis with passage into the maternal circulation by entering vessels that traverse the basal plate of the decidua.

The overall transplacental passage of AFP is accomplished by the bulk flow of AFP-containing fluids driven by a fetal-to-maternal hydrostatic gradient across the placental villous surface (253). Fetal arterial perfusion pressures are higher than those in the maternal intervillous spaces. Umbilical venous pressure is also significantly higher than the intervillous space pressure, providing support for the hydrostatic pressure gradient mechanism (254). Obviously, areas of discontinuity in the syncytiotrophoblast layer would provide even more surface area to facilitate such routes. Fibrinoid deposits are thought to further enhance passage by providing an additional matrix surface area for temporary AFP adhesion/attachment. The second pathway for AFP passage involves traversing the basal plate in which AFP gains access to the maternal arteries and veins of the decidua. Since Furth and Adinolfi (254) reported AFP fluorescent localization in placental cells, AFP might be transported into the cytoplasm of the trophoblast cells or it might be synthesized by these cells. These observations may explain why enhanced AFP transfer will occur in the presence of gross anatomical abnormalities of the placenta such as damaged placental villi or abnormal rupture of vessels passing through the basal plate, as occurs in placenta accreta (255). Such anatomical lesions of the placenta may underlie unexplained elevated AFP levels (see Figs. 4 and 6), which could be associated with perinatal loss, preeclampsia, and intrauterine growth retardation (256, 257). Thus, a major link to altered maternal serum AFP levels appears to be related to placental dysfunction or an immature state of the placenta (240, 241).

AFP As An Anticancer Drug Conjugate. The use of HAFP as a carrier for the delivery of anticancer drugs, as previously predicted (4), has increased since the mid-1990s (Table IX). Since some chemotherapeutic agents display low selectivity toward tumor cells, attention has been focused on the specific targeted delivery of cytotoxic compounds into cancer cells. The use of drug conjugates to antibodies (as with antibiotics, plant and bacterial toxins, etc.) has been hampered by immune reactions toward such preparations and rapid rates of clearance by the reticuloendothelial system. However, the high density of cell surface receptors for growth stimulatory factors on tumor cells provides a rationale for using growth factors and oncofetal proteins as drug-ligand carriers (74–77, 258). The HAFP receptors on nonproliferating normal cells have been shown to be 10-fold less in normal T-lymphocytes in comparison with lymphoma cells (73–76). Thus, HAFP has been shown to avidly bind and selectively enter tumor cells via a recep-

tor-mediated endocytotic pathway (259, 260). Successful conjugation of HAFP with numerous anticancer drugs has now been reported; such drugs include doxorubicin, daunomycin, calichemicin, carboxyphosphamide, bleomycetin, chlorbutin, cis-platinum, and methotrexate (261).

In vitro studies using anticancer drugs conjugated to HAFP displayed a highly selective antitumor cytotoxic activity toward human tumor cell cultures (262). The optimal AFP:cytotoxic drug conjugation ratio was shown to range from 1:3 to 1:5, resulting in higher cytotoxicity than for the drug alone. The success of the cell culture studies spurred development of *in vivo* tumor models employing murine leukemia and myeloid cells lines (263). Using HAFP conjugated to vinblastine and calichemicin, the therapeutic effect of AFP:drug conjugates in cell cultures and on solid tumors was much greater than that of unconjugated antitumor drug preparations. Such reports prompted the rapid development and use of metallic complexes of the phthalocyanines conjugated to HAFP. *In vitro* studies using aluminum and cobalt complexes of phthalocyanines linked to HAFP demonstrated 1000 to 50 times greater selective cytoxicity against T-lymphoblastoma and ovarian carcinoma cell lines respectively (263). Success of this metallic-drug conjugation will allow the use of photosensitizing therapy that involves light irradiation following uptake of the drug.

HAFP has been selected as a vector for specific delivery of anticancer drug conjugates to target cells *in vivo* as mentioned above; this strategy was based on the overexpression of AFP receptors on the surface of malignant cells versus the negligible incidence on normal cells (263, 265). Due to the bystander effect on surrounding cells, designing of drugs that target solely tumor cell populations is a principal objective in the field of cancer chemotherapy. As shown above, the use of AFP:drug conjugates is both safe and effective. However, the problem of multi-drug resistance (MDR) in cancer cells has also vexed the clinical oncologist. A strategy to overcome or bypass the MDR of the mdr-1 receptor/transporter has employed the aid of AFP-mediated targeting of tumor cells (262). In a human ovarian carcinoma and a breast cancer drug-resistant cell line, HAFP conjugated to doxirubicin increased the drug sensitivity 10- and 4-fold, respectively. Thus, penetrating the tumor cells via receptor-mediated endocytosis with HAFP as the vehicle raised the drug sensitivity in these antibiotic-resistant cell lines. However, the breast cancer cell line MCF-7 did not fully overcome or break drug resistance and this was attributed to other multifactorial agents such as overexpression of topoisomerase II, and other membrane proteins such as the MDR-associated protein (MRP) and P-glycoprotein (261). Overall, these studies demonstrated that the HAFP-conjugated drugs were highly efficient against human tumor cells *in vitro* with demonstrable drug resistance possibly due to the hyperexpression of membrane receptors/and/or transporters.

AFP Ligand Binding and Transport. The binding and transport of ligands has long been recognized as a major function of AFP during fetal development (Table IX). The multiple ligands that bind HAFP have been described in a myriad of reviews on the biology of this fetal protein (5–7, 16, 17). However, recent reports have expanded this list to include environmental agents that impact the human population at large. Such agents now include the phytoestrogens, dioxins, and the flavinoids. Binding of rodent and HAFP to the phytoestrogens has previously been discussed and the reader is directed to these references (6, 265, 267). Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD]) belongs to a class of highly hazardous environmental contaminants formed as a by-product of technological manufacturing processes. The polychlorinated dioxins and the many related compounds (polychlorinated dibenzofurans, biphenyls, and other aromatic hydrocarbons) are highly toxic and carcinogenic. Such compounds are also embryotoxic, teratogenic, neurotoxic, and immunotoxic in a multitude of target organs (i.e., thymus, spleen, lymph nodes, adrenal, thyroid, mammary, and sex accessory glands). This highly embryotoxic agent has been found to form a noncovalent, stable complex with HAFP in a 2:1 ratio (265). The apparent solubility of TCDD in water increases 10^5 fold after binding to HAFP and the injected water-soluble complex is less cytotoxic in normal mice than TCDD injected in oil suspensions. However, in human tumor cell cultures (DEM, MCF-7, and Hep G2), the AFP-TCDD complex was highly toxic, displaying 200 to 1400 times higher toxicity than TCDD alone. The complex also surpassed dioxin in cell selectivity. The authors of this report cautioned that AFP might facilitate TCDD transport into embryonic tissues and thus enhance its embryotoxic and teratogenic effects (265).

Recently, awareness has developed of endocrine disrupters in the environment due to chemical contaminants found in fertilizers, pesticides, polychlorinated biphenyls, and nutritional (beef-fattening) hormonal supplements. These agents have been proposed as possible causes for the present-day increased rates of endocrine-related cancer. Since many of these chemicals display estrogen-like ring structures, they were thought to contribute to the estrogen biomass (i.e., phytoestrogens) already present in the environment derived from plant and animal sources (266). Interest in plant-derived bioflavonoid (BFV) effects in regulating steroid hormone action in mammals has recently aroused concern. Studies of the BFVs for possible modulation of steroid-dependent breast and prostate tumor growth, as well as during fetal development, have justified further testing in this field (267). Unfortunately, pharmaceutical screening for these estrogen-like compounds often employs fetal or neonatal rodent models in which AFP avidly binds estrogenic steroids with a $K_d = 5 \times 10^{-9}$ M, in contrast to HAFP, which does not bind these compounds (268). A recent study has now shown that binding of AFP to BFVs is sufficiently high ($K_d = 5 \times 10^{-7}$ M) to suggest that flavonoids (naringenin, flavanones, quercetin, kaempferal, and flavonols) and isoflavonoids (daidzein and genistein) could affect estradiol and estrone binding to rat AFP *in vivo* when

present at dietary levels (268). These authors found that the 5, 7-hydroxyl groups in ring-A and 4-hydroxyl group in ring-B of the BFVs are important for binding to AFP. Such studies could aid in elucidating the molecular basis for recognition of flavonoids and estrogens by AFP. Finally, these findings also emphasize that the flavonoid levels in the diet need to be further considered in studies of xenobiotics and endocrine manipulations in both experimental and drug-screening programs.

AFP as a Growth Regulator. Mammalian AFP is a fetal tumor-associated (oncofetal) protein that first and foremost promotes growth in a variety of *in vitro* (cell) and *in vivo* (animal) models (6, 7, 269) (Table IX). The presence of AFP is known to be associated with the successful completion of term pregnancies in mammals, and even nanogram amounts have been reported in fetuses and newborns that were thought to lack serum AFP; hence, it appears that even minute amounts of AFP may still be necessary during human pregnancy (270, 271). However, knockout mice for AFP have not yet been developed and the final answer must await the test of scientific endeavor. Since AFP has been identified at every stage of development from fertilization of the zygote, through the blastula, gastrula, embryo, and fetal and newborn periods, it is likely that this fetal protein plays a crucial role in ensuring the successful completion of pregnancy in mammals. It may be just a coincident occurrence that AFP, as an autocrine factor, also enhances the growth of a variety of tumors under certain concentrations and conditions (269, 272–274). However, AFP and its derived peptides are also able to inhibit proliferation in instances where growth is involved; these include fetal development, cancer cells, frog metamorphosis, liver regeneration, and glucocorticoid-induced splenic growth (6, 7, 58).

In the last decade, reports have confirmed that AFP serves as a dual regulator of growth, capable of both enhancement and inhibition (115, 270, 275–277). The capability of both up and down modulation of growth and differentiation as a dose-dependent function of AFP has now been demonstrated in a multitude of cell types including placental, ovarian, uterine, lymphoid, epidermal, endothelial, testicular, breast, and liver (278–282) and in certain types of cancers (283, 309). However, elucidation of the cell regulatory mechanisms of these growth events involving AFP remains to be determined. Reports now indicate that AFP regulates growth by enhancing or by inhibiting apoptosis (cytotoxicity) in tumor cell culture assays, while in cases of AFP-induced growth inhibition involving signal transduction, the event results in a cytostatic rather than a cytotoxic effect (see Refs. below).

AFP can apparently regulate growth by several mechanisms, which might include apoptotic regulation, cytoplasmic signaling modulation, and receptor desensitization. Regarding apoptosis, it has previously been reported that hepatoma cells from the cell culture line HepG2, washed free of AFP that was bound to the cell surface, became susceptible to tumor necrosis factor (TNF) cytotoxicity (283). The HepG2-derived cells had previously been shown to be resistant to TNF cytotoxity. Subsequent studies revealed that HAFP could induce apoptosis in various tumor cell culture lines (heptoma and lymphoblastomas) in just a few hours (284). Induction of tumor cell death was shown at high physiological doses of AFP (>250 μg/ml), but not at low doses (<200 μg/ml). It is interesting that embryonic fibroblasts responded only by increased cell proliferation and not by apoptosis. The AFP-mediated apoptosis is mediated via Ca^{++} and tyrosine kinase-independent pathways and did not require protein and/or RNA synthesis (285). However, AFP-induced apoptosis in tumor cells (human lymphoblastoma Jurkat cells) was shown to be abrogated by endogenous or exogenous interleukin-2 (IL-2) and other immunoregulatory agents (286). Subsequent studies revealed that the AFP-mediated apoptosis occurred in a manner independent of the TNF receptor, and that AFP could mediate growth regulatory signals in TNF receptor-impaired cells (287). It was further determined that HAFP induced apoptosis in Raji tumor cells by activation of caspase-3 and, in turn, bypassed the FAS and TNF receptor-dependent signaling pathways (288, 290). The activation of caspase-3 proteases by AFP was also independent of upstream activation of the initiator caspase-1,8, and 9 proteases (291, 50). In summary, the cytotoxic effects of AFP are apoptosis-linked and are independent of the down regulation of the Bcl-2, Bcl_x-L proteins, and the TNF receptor-dependent signaling cascade.

HAFP has been reported to display growth regulatory activity on different types of tumor, as well as normal, cells. As discussed above (261), the presence of the AFP receptor on tumor cells and lymphocytes enables AFP to be readily endocytosed. In light of its regulation of apoptosis, AFP in high doses (>100 μg/ml) was found to exhibit *in vitro* growth inhibition on a variety of tumor cells including hepatoma HepG2, lymphoblastoma MT4, Jurkat lymphoma, murine fibroblastoma L-929, and MCF-7 breast cancer cells (292). An equivalent dose of human ALB had no effect. However, low AFP doses (<100 μg/ml) failed to inhibit growth in any cells tested and rather showed a slight stimulating effect. Interestingly, all AFP doses were growth stimulating to embryonic epidermal fibroblasts. These *in vitro* data demonstrate that AFP could induce either stimulative or inhibitory growth activity, depending on the relative concentration of AFP and of exogenous or endogenous cytokines, hormones, and growth factors in the culture media. Subsequent studies employing an estradiol- (E_2) induced conformational change of the AFP molecule further resulted in significant growth suppression of MCF-7 cells (292). Low doses of AFP (0.01 μM) incubated in molar excess of E_2 (but with AFP:E_2 preincubation) produced the suppression, but AFP and E_2 added to cultures without preincubation induced only apoptosis leading to cytotoxicity in these cells.

It has previously been documented that HAFP could display an antiestrotrophic property following incubation

with high concentrations of E_2 or other ligands of the steroid receptor superfamily (115, 294, 295). It was subsequently demonstrated that ligand incubation of HAFP was not required when 100-fold increases in HAFP doses were employed (from 1.0 μg up to 100 μg) in the inhibition of an E_2-dependent human breast cancer xenograft (201). Such data can be interpreted to propose that a small percentage of HAFP already exist in a conformationally transformed (inhibitor-exposed) variant form. These findings led to studies to determine what active site on HAFP was responsible for and harbored the antiestrotrophic active site (87). A recent report employing a recombinant portion of HAFP confirmed that this activity localized to the third domain of HAFP (295) in E_2-dependent, but not -independent tumors. Although daily administration of the HAFP was required for 30 days, both the MCF-7 and the T-47 cancer cell strains were almost completely growth-inhibited. A highlight of this study was a demonstration that the native HAFP, as well as the third domain of HAFP, underwent a time-dependent spectrum change when analyzed in the presence of high E_2, confirming a conformation-induced change in these molecules. The transformation of HAFP by ligands such as E_2 (292, 293) confirms similar changes induced by fatty acids (C18 to C22), which also bind AFP (8). One active site exposed following an AFP conformational change has been detected, and the peptide has been synthesized and purified as been described above (see Ref. 87).

A second method that AFP could employ to regulate growth might involve protein-to-protein interaction in the course of cytoplasmic signaling cascades (297). Since AFP has not been reported to localize in the intranuclear compartment of cells (296), it is unlikely that AFP can directly affect the nuclear transcription process. In contrast, AFP has been localized in ECMs, at cell surfaces, and in receptosomes, endosomes, the ER, and perinuclear spaces within the cytoplasm (202, 259, 75). A nonsecreted form of AFP has also been reported (45–47). Therefore, it is conceivable that AFP could interact with receptors, binding proteins, transcription factors, kinases, coactivators, and cell cycle regulators in the cytoplasm prior to their nuclear translocation. For example, AFP binding to a co-activator or a transcription factor might mask a nuclear localization signal (zip code), essentially impeding its cytoplasmic-to-nuclear translocation; alternatively, AFP fragments or truncated forms could serve as short heterodimer partners to dimerize and neutralize such factors (47, 48).

Mitogenesis induced by estrogen could serve as a paradigm for hormone-induced growth, since rodent and HAFP have been demonstrated to regulate E-sensitive growth (298). E_2 stimulation of quiescent MCF-7 breast cancer cells results in E_2 binding to the estrogen receptor in the cytoplasm and/or nucleus. The E_2-bound estrogen receptor reacts with DNA at the palindromic estrogen response element (ERE), resulting in initiation of the transcriptional activity of immediate-early genes (299). Following gene activation, the expression of the cyclins and their associated proteins ensues, resulting in G_0 to G_1 cell cycle progression via cyclin D1 and the CDK4 and CDK6 kinases (299). However, recent reports now show that various elements of the cytoplasmic signaling cascades are also E-responsive. In bone cells expressing estrogen receptors, the mitogen-associated protein kinases (MAPK) family members of ErK-1 and ErK-2 (extra cellular signal-regulated kinase) are activated within 5 min following E_2 stimulation (300). The cytoplasmic signaling data are further supported by reports of the existence of cell membrane-associated estrogen receptors that are G protein-coupled (301). Activation of MAPK indicates that at least some part of the mitogenic stimulation by estrogen may be mediated through a cytoplasmic pathway.

Recent studies have suggested that MAPK activation may actually be a requirement for estrogen-induced cell cycle progression (297, 300). These reports showed that MAPK and phosphatidylinositol 3-kinase (P3K), but not PKA or PKC (protein kinases), were obligatory for E-stimulated cell cycle progression. The use of MAPK and P3K inhibitors showed that a cell cycle block occurred just after the expression of immediate/early genes and prior to the induction of cyclin D1 expression, resulting in G_1 arrest (299). Thus, it is conceivable that intracellular-bound AFP, either as the endocytosed, nonsecreted, or the estrogen receptor form, may be capable of blocking mitosis stimulated by E_2 and possibly other growth factors.

The third method by which AFP and/or its derived peptides could modulate cellular growth might involve the interruption of G-coupled signal transduction in a process termed "agonist desensitization" (304). Unlike the cytoplasmic signaling discussed above, this signaling cascade occurs at or proximal to the cell membrane. Following binding to cell surface G-coupled receptors, endocytosed clathrin-coated receptosomes are generated. The endocytotic clathrin pathway attracts a G protein-associated adapter protein termed arrestin and the mechano-enzyme, dynamin (305, 306). Arrestin binding to the receptor uncouples the G proteins and no further kinase phosphorylation cascades occur (307). Clathrin is bound by arrestin and assisted by dynamin in trafficking the receptosome protein/peptide cargo to either an endosome or proteosome destination. Dyamin mediates the fission step subsequent to the initial coated-pit formation in the membrane (vesicle) recycling process (306). However, rapidly repeated agonist stimulation or high agonist concentrations depletes the cell surface receptor population (without replacement), which renders the cell insensitive to further stimulation.

The G-coupled receptors are seven-transmembrane domain proteins linked to G protein extracellular receptor kinases (ERKs), which respond to neurotransmittor, hormone, and growth factor stimulation (303, 307). Signal transduction of this receptor type can thus be uncoupled by the "desensitization" process described above. In this process, a blunting of the second messenger responses occurs following prolonged or excessive agonist exposure (308, 309).

AFP and its derived peptides might desensitize or quench G-coupled receptor-mediated signal transduction pathways and functionally impair the cellular growth response of multiple cell types including tumor cells. Thus, AFP/and its derived peptides might serve as decoy ligands for G-coupled receptors, which could uncouple ERK-protein cascade interactions. This, in turn, might affect the mitogenic response to MAPK interactions, which are involved in cellular proliferation. Such a pathway would represent a blockade of growth factor receptors, thereby depriving the cell of further proliferation.

Cancer Chemoprevention. The recent epidemiologic observations that high AFP levels in pregnant women reduce their subsequent risk of both pre- and postmenopausal breast cancer may be related to the growth suppressive properties of AFP (Table IX). An initial study by Richardson *et al.* (327) has indicated that a reduced risk of postmenopausal breast cancer is associated with high third trimester AFP levels in women younger than age 28 at first pregnancy. A subsequent report by Melbye *et al.* (328) confirmed and extended these findings to include second trimester AFP blood values in premenopausal women up to age 38. Thus, both studies concluded that high levels of AFP in maternal serum during any pregnancy were associated with a low incidence of breast cancer; the association was particularly strong for pregnancy at young maternal age. These results provide the impetus to propose that AFP and its derived peptides (173) could be employed not only for cancer therapy, but also for the chemoprevention of breast and possibly other tumors as well (329). This protective effect of AFP during pregnancy was also observed in women bearing multiple pregnancies (twins), neural tube defects, and presenting with pre-eclampsia (330–332). All these situations share a commonality of elevated serum AFP levels, which may contain sufficient quantities of AFP conformationally induced variants to affect growth suppression of cancer microfoci present in the maternal breast tissues (173, 309). The presence of cancer microfoci in the young adult and the middle-age population has been described (J. Folkman, M.D., personal communication).

Concluding Remarks

In the course of this updated review on AFP, the human form of this fetal protein has been the center of focus. This emphasis on HAFP is a direct reflection of the biomedical literature of the last 5 years. In the 1970s and early 1980s, animal forms of AFP were abundantly identified, purified, and characterized; these included rabbit, mouse, rat, guinea pig, cow, sheep, goats, pigs, and monkeys. Although HAFP was first purified in 1970 (See Ref. 6), its isolation continued to be difficult due to its copurification with serum ALB. When the HAFP gene was cloned in the early 1980s and HAFP immunoassays were approved for clinical use in 1984, a trend developed in the scientific literature toward an increase in the publication of human clinical applications. This trend was propelled by the advent of MoAbs for direct application measurement of HAFP as a diagnostic marker in screening for fetal birth defects and various tumors. However, during this period, reports of different AFP isoforms and heterogeneic variants continued to emerge with additional nomenclatures. Concomitantly, funding for basic research on AFP in the United States drastically declined when clinical usage for fetal defects and cancer became routine nationwide.

This overview has, therefore, attempted to update the reader concerning the classification, nomenclature, and status of the various isoforms, epitopes, peptic fragments, and conformational variants exhibited by HAFP (Table X). Such forms should be considered in the design of immunoassays, purification strategies, physicochemical studies, genetic variants, and bioassays for HAFP. As proposed in 1985 (4), HAFP has also been employed as a carrier vehicle for anticancer drugs and for the biomodulation of both ontogenic and oncogenic growth processes. The cloning of the HAFP gene and its translated protein product have further enabled the use of short specific peptide segments obtained

Table X. Summary of Mammalian Alpha-Fetoprotein Isoforms and Variants Described in the Present Report Regarding Number and Mode of Detection

Source of AFP isoforms and variants	Mode of detection and analysis	Number identified	Manuscript section	References
A. mRNA transcripts of translated proteins	Cloning, sequence analysis, nuclear acid hybridization, nucleases, PCR	8	2A	31–42
B. Free/bound forms	Immunological physicochemical	9	3A, B	4, 44–55, 65–77
C. Peptide fragments	Enzymatic cleavage, peptide synthesis	26	4A, B	86–90
D. Cell adhesion	Genebank, adhesion plate assays	8	5A, B	91–111
E. Carbohydrate, N-linked glycans	Chromatography, electrophoresis, lectin affinity	10	6A	118–133
F. pH isoforms	Electrophoresis, isoelectric focusing, chromatofocus	3	6B	137–142
G. Antigenic epitopes	Monoclonal antibodies, epitope mapping	6–8 (major) 12 (minor)	7B, C, D	158–172
H. Molten globule intermediates	Fluorescence, circular dichroism, microcalorimetry	2–3 (?)	8B, C	175–178

Note. AFP, alpha-fetoprotein and N, asparagine linked.

from recombinant and synthetic peptide technologies as probes for biological activities, subcellular trafficking, and therapeutic targets. With the documentation of AFP and its derived peptides as regulators of growth, studies of carrier vectors for genetic manipulation and histocompatability modulation are now being pursued. It can only be expected that more imaginative and creative uses of AFP and its derived fragments will be employed as the future dictates. The ongoing euphoria regarding the success of the Human Genome Project at the DNA level will inevitably give way to a recognition of the enormity of the next challenge, Proteomics. The structure and function of the AFP, as delineated here and in previous reviews, is a particularly good example of this future challenge.

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