MINIREVIEW

Biological Roles of Alpha-Fetoprotein During Pregnancy and Perinatal Development

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The use of alpha-fetoprotein (AFP) as a serum marker in cancer actually predates its employment in the detection of congenital defects; however, the latter use of AFP as a fetal defect marker has propelled its clinical utilization. Although the serum-marker capacity of AFP has long been exploited, less is known of the biological activities of this oncofetal protein during fetal and perinatal development. In the present review, the biological activities of AFP are discussed in light of this glycoprotein's presence in various biological fluid compartments: embryonic and fetal tissues, serum, urine, and reproductive fluids. After a review of the histochemical detection of AFP in various cells and tissues during development, AFP concentrations within various biological fluids were discussed in the context of gestational age and anatomic location. Discussion follows concerning the relationships and roles of AFP in developmental events such as erthyropoiesis, histogenesis/organogenesis, and ligand binding and in developmental disorders such as hypothyroidism, folate deficiencies, and acquired immunodeficiency disorder (AIDS). Based on its association with so many types of birth defects, malformations, and congenital anomalies, AFP can be viewed as a molecular "troubleshooter" until signal transduction pathways are established during pregnancy and prenatal development. The review concludes with a discussion of the place of AFP in the rapidly expanding field of proteomics. Exp Biol Med 229:439–463, 2004

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Introduction

Historical. A fetal component not commonly found in adults was first detected as a postalbumin migrating protein in fetal serum by Bergstrand and Czar in 1956, using paper electrophoretic techniques (1); subsequently, Masopust and Kotal assigned to the unknown developmental protein of Bergstrand and Czar the name "fetoprotein" (2). Gitlin and co-workers (3) then devised the name "alpha-fetoprotein" (AFP) for the electrophoretic α_1-migrating human fetal protein. In 1963, Abelev and co-workers (4) reported, in hepatoma-bearing mice, a protein that migrated in the α_1 region of an electrophoretogram, and in 1965 Tatarinov described a similar protein in the sera of humans bearing hepatomas (5). In the early 1970s, Brock and co-authors/co-workers reported elevated AFP levels in human amniotic fluid (6) and in maternal serum (SAFP; Refs. 7, 8) that correlated with the presence of neural tube defects in the fetus. Thus, studies involving the α_1-migrating fetal protein as a gestational age–dependent fetal defect marker actually postdated its recognition as a tumor marker, and the name "oncofetal protein" was assigned (9).

Mammalian AFPs are single-chain glycoproteins with molecular masses ranging from 66 to 72 kDa and a 3%–5% carbohydrate (glycan) content (10–12). Alpha-fetoprotein is a tumor-associated fetal protein classified as a member of a three-domain albuminoid gene family that currently consists of four members: albumin (ALB), vitamin-D binding protein (DBP), AFP, and alpha-albumin (α-ALB; Refs. 13, 14). Similarly to ALB, SAFP is known to bind and transport a multitude of ligands, including bilirubin, fatty acids, retinoids, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxins, and various organic drugs (15, 16). Unlike ALB, high concentrations of hydrophobic ligands (i.e., fatty acids, estrogens) have been reported to induce an

irreversible conformational change in the tertiary structure of AFP (see Ref. 17 for review). Altered SAFP levels have been observed concurrent with aberrant growth manifestations, but it was usually assumed that these AFP levels were coincident events rather than the cause of such changes. Although AFP may not be the direct cause of the altered growth manifestations observed in birth defects, it is conceivable that some shock/stress-induced conformational (variant) forms of this fetal protein influence, modify, or contribute to such events. Over the past decade, reports have emerged that some of these AFP forms can serve as dual regulators of growth, capable of both enhancement and inhibition of growth, in cancer as well as fetal cells (18, 19). The growth regulatory property of AFP is one of the most prominent characteristics that distinguishes this fetal protein from serum ALB.

Objectives

To date, there exists a paucity of published reports in the biomedical literature addressing the biological activities of AFP during fetal development. Moreover, the physiological roles of this oncofetal protein in the regulation of growth and differentiation during mammalian development have not been recently reviewed or updated. The objectives of the present review are 4-fold. First, the various uses of AFP, as an investigational probe and/or marker during development, especially in experimental settings, will be described. Second, the relationship of AFP's biological (physiological) activities in experimentally induced mammalian (including human) growth/differentiation models will be presented. Third, the multitude of congenital malformations, disease states, and biological activities ascribed to AFP in recent years justify a review that links the presence of developmental anomalies of AFP with physiological status. Finally, the biology of AFP during growth and development will be emphasized; many prior reviews have focused on AFP only as a diagnostic fetal defect marker. For a more extensive exposure to the physical chemistry and genetics of AFP, the reader should consult previous reviews (20–24). The present review is also intended to serve as an update and extension of previous reviews on AFP published in this journal (16, 17).

Ontogeny of AFP

The original observation that AFP was synthesized by a variety of mammalian tissues prior to and following parturition stemmed from the pioneering studies of David Gitlin (25). In 1972, Gitlin and co-workers demonstrated that AFP was synthesized by fetal liver and yolk sac; subsequent cell culture studies by his group indeed demonstrated that AFP was synthesized by a multitude of tissues, especially those of gastrointestinal origin (26). The detectability of AFP during ontogeny precedes the detectability of ALB by a considerable period of time. During the rodent 21-day gestation period, ALB is not synthesized until Days 12–13, while AFP is detected by Day 6 following fertilization and implantation (27). Moreover, AFP synthesis in rodents is initiated by events prior to and during implantation. In the mouse, AFP (MAFP) has been histochemically detected in the inner cell mass of the blastocyst, in both the outer and the inner layers of the primitive endoderm (28, 29). The outer endodermal layer gives rise to the parietal endoderm, while the visceral endoderm emerges from the inner endoderm layer. Bovine AFP (BAFP) has also been detected in the 14-day trophoblast, and by Day 16, BAFP is secreted into the amniotic fluid (30). Similar to the AFPs of marmosets and rodents, BAFP is detected in both the preimplantation and the postimplantation conceptus (31). In summary, all mammalian species studied thus far show histochemical evidence of AFP in the pre- and postimplantation embryos, yolk sac, amnion, embryonic disc, and early primitive streak stages.

The synthesis of AFP in the liver anlage (primordium) and other embryonic tissues has been extensively studied through analysis of expression patterns of AFP mRNA in both human and mouse embryos (36). Human AFP (HAFP) is expressed in the yolk sac, hindgut/midgut endoderm, and the foregut hepatic diverticulum at 26 days postovulation. At 32–52 days postovulation, HAFP was found to be strongly expressed in the mesonephric duct and tubules; however, HAFP was only transiently expressed in the pancreas at 40–50 days. Although HAFP was not expressed in the metanephric kidney, expression was apparent in the bile duct and gallbladder endoderm.

In comparison, MAFP mRNA is expressed in the primitive hepatocytes of the hepatic buds of 9.5-day embryos (27). At this stage, MAFP expression was also observed in midgut and hindgut endoderm up to Day 13.5. As seen in the human embryo at a corresponding stage, pancreatic expression was at best weak and transient only, during the Day 11.5–Day 13.5 period. In contrast to the human pattern, MAFP expression was not detected in either the mesonephric or the metanephric kidneys.

AFP in Developing Brain

The presence of AFP in tissues of fetal brain was first described by Gillin and co-workers (5) in culture studies employing tissues of the human conceptus (26). Later, in murine brain cytosols, it was reported that the major estradiol binding protein in the fetal brain was actually AFP, a protein synthesized in liver and yolk sac (32). Human fetal brain tissues obtained at autopsy and stained by immunohistochemical procedures revealed positive AFP staining in nerve cells of the cerebral wall, brain stem nuclei, and the epithelial layers of the choroid plexus (CP; Ref. 33). The presence of AFP within cells of the CP suggested that the fetal protein was transudated from the blood to the cerebrospinal fluid (CSF) *via* a cellular route across CP epithelial layers. These observations prompted the proposal that presence of AFP in

the brain plays a role in neuronal differentiation and/or development. Indeed, the localization of AFP and other serum proteins in various neurons of the developing mouse brain was confirmed by means of immunohistochemical methods throughout the 1980s (34–45).

Immunohistochemical procedures were further used to document that AFP was present in developing rat and mouse brains throughout fetal and postnatal development and for up to 20–25 days following birth (34, 36, 39, 40). Large tissue areas and groups of cells in many regions of the developing brain, from the olfactory bulb to the medulla oblongata, stained positively for AFP at various time intervals during development. Intracellular labeling localized AFP to the cytoplasm of the neuronal cells and extending into these cells' axonic and dentritic extensions. In human brain tissues, peak levels of brain AFP were detected in fetuses up to and including the 20th week of gestation (41, 42). However, no AFP could be detected in fetal brains obtained from the third trimester of human pregnancies. In sheep fetal brain homogenates, histochemical detection of AFP was reported prior to 60 days' gestation in many immature neurons in the neuroependymal layers and in several layers of the developing cortical plate (35). In like fashion, AFP was localized in fetal pig brain tissues representing the ventricular ependyma, meningeal envelopes, CP, and blood vessel walls of the brain (37). As seen in humans, porcine AFP was found only in the cytoplasm of differentiating neurons at the axonal pole of the cell and in the dendritic processes of pyramidal cells (43). Studies in the 9-week-old fetal baboon brain further demonstrated the presence of AFP in neural tube and neural crest derivatives and in the ventricles, which displayed an intracytoplasmic staining pattern (38). The staining localization of baboon AFP appeared to decline as myelination and glial-cell development progressed. Uriel and co-workers proposed that the binding and transport of polyunsaturated fatty acids by AFP (see Ref. 44) could explain the presence of this fetal protein in the developing nervous system (see the section "AFP and Proteomics"). Studies in human embryos further showed immunohistochemical localization of AFP to in the lateral ventricular zones of the fore-, mid-, and hindbrain (41, 42).

By the early 1980s, it had become evident from published reports that innate intracellular synthesis of AFP in brain cells was unlikely. Although AFP and other serum proteins were found intraneuronally in developing mammalian brains, observations showed that local (brain) production, of either AFP proteins or the mRNAs coding for them, could not account for this localization (45). These data supported the concept of a nonbrain origin of AFP. Indeed, later reports confirmed that radiolabeled AFP was actually taken up and incorporated into developing brain cells undergoing neuronal differentiation (44). The evidence against the synthesis of AFP in rat brain cells was further strengthened by reports that no mRNA transcripts for AFP could be found in fetal brain and heart tissues, in contrast to the situation in fetal intestine, lung, liver, and kidney tissues (46). However, both AFP and ALB gene transcripts have since been reported by *in situ* hybridization, in various rat tissue sections, using S^{35}-labeled AFP and ALB cDNA probes (47). In that report, AFP and ALB mRNAs were found to be present in distinct cell populations of developing rat brain and kidney tissues. Cellular transcripts were localized to the cytoplasm during fetal/postnatal life and were found in the nucleus at 3–5 weeks, suggesting that post-translational mechanisms are involved in the control of stage-specific AFP/ALB gene expression.

AFP and Homeodomain Proteins

The discovery of the homeobox transcription factor gene family remains a hallmark discovery in embryonic development and differentiation (for review, see Ref. 16). These embryonic inducers, first discovered in insects, are now known to exist as homeotic proteins in nematodes, plants, yeast, rodents, and human beings. Homeoproteins serve to direct and control pattern/positioning body development in embryos regarding anterior/posterior, trunk-thorax segmentation, dorsal/ventral axis, body/cell polarity, neural tube formation, and caudal/gut formation. For example, birth defects are often homeotic transformations resulting in developmental abnormalities in which one part of the body develops in the likeness or dissimilarity of another. Pattern formation in the embryonic germ layers usually involve a network of feedback systems between intrinsic factors of gene expression in developing precursor cells and extrinsic signals exerted from the surrounding embryonic matrix environment (16, 17). The homeotic proteins frequently modulate or mediate inductive pathways that partition early axial germ layers into structures or segments with distinct regional identities. These morphogenetic processes are then linked to the terminal differentiation stages of that particular germ-layer derivative. Examples of the homeodomain transcription proteins include Pou, Crumbs, Hox, Antennepedia, Wnt, Sonic Hedgehog, Notch, and Pax (16). Human AFP itself appears to contain amino acid sequence identity/similarity stretches to the homeodomain proteins on all three of its domains (Table 1).

Since the homeodomain proteins are present during early embryogenesis, it would seem reasonable that AFP might display short homeodomain sequences in molecular mimicry of these pattern-regulating proteins. For example, mutations in the Pax-3 domain result in central nervous disorders relevant to AFP such as anencephalies and spina bifida, in addition to abnormalities associated with neural crest structures (16). In mammals, Pou domains are expressed during early embryogenesis in many regions of the developing brain including forebrain and nerve cord (Table 1). Aside from binding the major groove of DNA, the Pou domain is required for homo-and heterodimerization of the Pou domain proteins. The Pit-1 gene of the Pou domain controls development of the anterior pituitary, and muta-

Table 1. Genbank Amino Acid Sequence Matching of Human Alpha-Fetoprotein (HAFP) Domains 1, 2, and 3 with Conserved Sequences from Various Homeodomain Protein Segments.[a,b]

		Percent (%) identity/similarity	Percent (%) total
I. Homeodomain signature (HS) sequences			
(A) Amino terminal HS	MXSYP	100/0	100
$HAFP_{266}$	MXSYI/C	75/25	100
(B) Carboxyterminal HS	YXPWM	100/0	100
$HAFP_{502}$	YVPPF	40/40	80
II. Domain 1			
Amino acid region 5–19			
Human AFP	NEYGIASILDSYQCT	100/10	100
Murine Pou domain	QXYGTHSYLHTYVCT	40/20	67
III. Domain 2			
Amino acid region 223–237			
Human AFP	KLSQKFTKVNFTEIQ	100/0	100
D. melano. notch	MXNVKFDNVRFVEIT	50/7	57
D. melano. Antennapedia	KLPAKFTTXNFELF	50/21	71
Amino acid region 239–253			
Human AFP	LVLDVAHVHEHCCRGDVLDCL	100/0	100
D. melano. Pou domain	LVAHGIHVHGGCCSGCYAGCL	52/14	66
Amino acid region 250–268			
Human AFP	CCRGDVLDCLFDGEKIMSY	100/0	100
Xenopus Hox/7a	CCPGDAASSXEGAERLHSY	39/27	66
IV. Domain 3			
Amino acid region 445–456			
Human AFP	LSEDKLLACGEG	100/0	100
Porcine Hox 2.4	CSICELLYCGEG	58/8	66
Amino acid region 471–480			
Human AFP	EMTPVNPGVG	100/0	100
Human crumbs	QWTPVNPGVQ	70/10	80
Human Pax-3/FHI	IMTPVDPGVP	70/10	80
Human Hox-G2	EMTPSTPGLQ	60/30	90
Murine Wnt-7a	QHTPVRPGVA	60/20	80
Rattine notch	EMQPLRPGAS	50/30	80
Rattine Pou domain	QKTPIFHCHT	20/40	60
Amino acid region 511–552			
Human AFP	KFIFHLDLCQ	100/0	100
Rattine Pou domain	SFIFXFNLCQ	66/22	88

[a] Note that the Pou domain proteins, which are involved with brain development, are present on all three domains of HAFP. Moreover, mutations of the PAX homeodomain protein have been linked to central nervous disorders such as anencephalies and spina bifida disorders (HAFP region 471–480).

[b] HAFP, human alpha-fetoprotein; *D. melano., Drosophila melanogaster;* antennapedia, crumbs, Hox, notch, Pou, and Wnt are all homeodomain proteins. HS, homeodomain consensus sequence as published in Ref. 236.

tions of this gene display failure of adenohypophysis development. The Wnt gene codes for proteins that are expressed in the midbrain-hindbrain border, and mutations in this gene results in the absence of these brain regions. Finally, Crumbs protein mutations have led to severe disorganization and degeneration of ectodermally derived embryonic epithelia. Thus, it may be more than a coincidence that HAFP segments share short amino acid sequence homologies with the homeodomain proteins, which are endowed with embryonic body positional information.

AFP Levels in Urine

The detection of AFP in human pregnancy urine was first described by Smith and co-workers in 1971 (48); AFP was detected in the urine of hepatoma patients in that same year (49). Due to the lack of sensitive immunoassays, the samples were scored as positive only for highly concentrated urine specimens; however, AFP mass values were still considerably lower than in serum. Later in 1973, AFP was detected in the urine of pregnant and hepatoma-bearing rats, but not in normal adults, by means of Ouchterlony immunodiffusion (50). In the gestating rat, AFP is produced in the fetal liver and yolk sac, secreted into the fetal circulation and amniotic fluid, passaged into the maternal circulation *via* the placenta/allantois, and excreted in maternal urine. In the rat, AFP is reportedly excreted in the urine for at least 6 days postpartum (51). After intrauterine death and resorption of the rat conceptus, urinary excretion of AFP persists for several days, while

remaining signs of pregnancy regress to nonrecognizable levels; nevertheless, AFP is still detected in the urine. Excretion of AFP into urine can also be detected in very early stages of chemically induced carcinogenesis, in rats fed carcinogenic diets containing carbon tetrachloride (52). In rats fed 3-methyl-4-dimethylaminoagobenzine, AFP in urine was detected in 90% of the experimental animals. Readers are further referred to a recent publication (53) by the author for an updated review of AFP in pregnancy biological fluids including urine.

AFP in Reproductive Fluids

Alpha-fetoprotein has long been detected in the biological fluids of the reproductive tract. The mammalian oviduct secretes, by transudation and exudation, a variety of fluids that may influence multiple reproductive activities, including sperm function. Such reproductive fluids, which include cervical mucus, oviductal fluid, and follicular fluid, can facilitate sperm capacitation (aging/maturation), fertilization, zygotic cleavage, and early embryonic nourishment (54–56). Electrophoretic studies of oviductal fluids from rabbits, monkeys, and women have demonstrated that the mammalian oviduct secretes a variety of proteins such as ALB, β-globulins, and AFP (54, 55). Earlier studies provided evidence that the production of proteins found in human oviductal fluid (HOF) is under hormonal control and that levels of such protein secretions correlate with the estrogen peak of the menstrual cycle (56). Since the proteins appear to be estrogen regulated within the oviduct, they may have functional similarities while differing in molecular mass. The proteins derived from HOF have two sources: (i) proteins originating from serum transudation and (ii) those synthesized and secreted by the uterine tubal mucosa (55). One such secreted protein, designated as "human oviductin-1" (HOV-1), displayed a molecular mass of 54 kDa and a PI of 4.5 and contained a carbohydrate moiety (56). Subsequent studies in rabbits detected a similar protein in the post-ALB range of an electrophoregram and showed evidence of induction by 17β-estradiol while demonstrating Periodic Acid Schiff (PAS)–positive staining (55, 56). The 54-kDa human oviductal protein contained 8% carbohydrate (vs. 3%–5% in SAFP), showed isoform heterogeneity, and did not react with anti-ALB antisera. The human HOV-1 protein was of nonserum origin and did not cross-react with extracts of human liver, kidney, or ovary, but it did react against human oviductal tissue. Studies using tritiated leucine confirmed that the protein was a secretory product of oviductal origin. Furthermore, reducing SDS gels indicated a molecular mass of 54 kDa, while the same protein displayed a molecular mass of 66 kDa in non-reducing gels. Thus, the protein appeared to be a heavily glycosylated, AFP-like molecule. At the time of publication, the authors had not ruled out immunological cross-reactivity with α-ALB (human afamin).

In subsequent studies of the HOV-1 molecule, fresh donated human sperm were incubated with (i) a mixture of HOF-specific proteins or (ii) HOV-1. Using indirect immunofluorescence, the investigators studied the ability of the HOF proteins to bind to the human sperm (57). While the mixture of HOF proteins bound diffusely over the entire surface of the sperm, cell HOV-1 binding was restricted to only its head region. The authors stated that the HOV-1 protein acted as an acrosome-stabilizing factor, serving to prevent premature acrosome activation. However, they were unable to differentiate between capacitation and the acrosomal reaction in their sperm populations. In subsequent purifications of HOV-1 protein, determination of the amino acid and carbohydrate compositions of HOV-1, together with isofocusing and Western immunoblot analysis using monoclonal anti-HAFP antibodies, confirmed that HOV-1 was antigenically identical to HAFP (58). However, its molecular mass suggested that HOV-1 is a truncated form of AFP (17). Thus, HOV-1 was a globular, non-collagenous protein with carbohydrate attachment *via* an N-glycosidic linkage between N-acetyl-glucosamine and asparagines, as is the case for HAFP. The authors proposed that HOV-1, like AFP, was secreted into the luminal spaces of the oviduct as a result of AFP biosynthesis by mucosal cells of the Fallopian tube (58–60). Since total HOF has been shown to prolong sperm survival, AFP as a constituent protein may serve to mediate sperm survival and motility. In the same study, HOV-1 was found in the HOF of a patient during the periovulatory period (59, 60). Interestingly, AFP has been detected in the follicles of rats prior to and during the ovulatory cycle (15). The detection of HAFP in postovulatory stages of the human ovum lends credence to a role for AFP in reproductive-tract physiology (28, 29). Overall, female reproductive tract secretions *in vitro* have been shown to maintain follicular fluid-induced hyperactive sperm motility while simultaneously decreasing the response of the acrosomal reaction so as to prolong sperm viability/function (58, 60). It is germane to this discussion that AFP binds to the male antifertility drug gossypol, which inhibits estrogen binding to AFP (61). Thus, AFP may serve a role in the fertilization process that is not yet fully understood or appreciated (see below).

In a recent study, a "knockout" of the AFP gene in mice was reported by a group of investigators in Belgium (62). They used gene targeting to show that AFP is not absolutely required during embryonic development, at least in the mouse. The AFP-null embryos developed normally, and transplanted embryos homozygous for the AFP-null trait developed normally, in an AFP-deficient microenvironment. However, while mutant homozygous adult male offspring appeared to be viable and fertile, AFP-null females were infertile. These investigators determined that the female infertility defect was related to a dysfunction in the hypothalamic/pituitary-ovary axis, as previously proposed (63). Although AFP does not seem to be required during development, due to compensatory actions of ALB and α-ALB, it plays a critical, nonredundant role in

determining the future fertility of female offspring in the mouse. In light of the reported presence of AFP in the developing follicles of the ovary, in HOF, and in the developing and newborn brain, AFP may somehow be involved in the maturation and programming of the positive feedback exerted by ovary-derived estrogen on brain LH and FSH levels. Indeed, earlier studies in rats showed that the postnatal decline in SAFP was strongly linked to the progressive increase in tissue-to-serum ratios of estradiol E_2 during the first 5 weeks of life (64). During these postnatal weeks, AFP and FSH levels declined, and an LH surge developed in a progressive, stepwise fashion (65). If AFP is injected during this period, it causes a significant rise in plasma FSH, low levels of free E_2, and a delay in the onset of puberty. These symptoms mimic the polycystic ovary syndrome in humans, in which anovulation persists in the presence of reduced FSH levels, concomitant with increased LH levels. The Belgian investigators suggested that the lack of AFP in null mice might mimic the polycystic ovary syndrome in humans and that further study was required to elucidate these issues (see Ref. 76).

AFP Antibodies During Development

The effect of heterologous or autologous antibodies to rat AFP (RAFP) in living systems has been extensively investigated. The presence of anti-autologous AFP antibodies in pregnant rodents was effective in interrupting development in a significant proportion of embryos (66–68). Using rabbit antiserum to rodent AFP, it was shown that the number of litters born was smaller, the birth weights were lower, the occurrence of stillbirths was higher, and the postnatal mortality was increased. Earlier studies had also documented a fetotoxic effect of heterologous anti-AFP antibodies in pregnant rats (66), whereas passive immunization of mice to AFP was only partially effective in fetal growth disruption (69, 70).

Antibodies to MAFP have likewise been employed as investigational probes and tools in the study of possible physiological roles for AFP. As previously observed in the chicken and in the rat, heterologous antibodies to AFP administered to pregnant mice resulted in developmental arrest, congenital abnormalities, hemorrhagic placental lesions, and fetal wastage (71–74). The cause of fetal death was linked to an antibody-induced anaphylactoid (Schultz-Dale) reaction of the uterus and antibody-mediated inflammatory lesions at the placental interface (73). It was readily apparent from these studies that breakdown of immune tolerance to AFP during pregnancy was hazardous to the completion of a full-term pregnancy. In a similar fashion, immune tolerance can be broken, as reported in pregnant rats, mice, and rabbits (75, 76). Finally, the administration of heterologous antibodies to MAFP in neonates in their first week of life resulted in characteristics resembling ovarian androgenization (i.e., polycystic ovaries), as discussed previously (76).

Uptake of AFP by Developing Cells

The uptake of AFP (endocytosis) was first observed in fetal cells using both *in vitro* and *in vivo* systems (77–82). The uptake or endocytosis of AFP was reported in cells derived from ecto-, meso-, and endodermally derived tissues of the embryo and fetuses of both birds and mammals (83). Subsequent studies showed the ability of cells of muscle origin to internalize exogenous AFP during fetal, neonatal, and tumor development (84, 85). The AFP is taken up by cells undergoing differentiation, whereas neither undifferentiated nor fully differentiated cells readily incorporate AFP. Furthermore, experimental evidence indicated that the entry of AFP into cells occurs by a process of receptor-mediated endocytosis (85–88). Following uptake, the AFP was first detected in clathrin-coated pits of the cell membrane; it thereafter progressed to vesicles, multivesicular bodies, and the trans-Golgi network surrounding the nucleus (89, 90). A series of subsequent studies further showed that AFP uptake also appeared in fetal cells of various species and origins, such as human rat, mouse, chicken, and baboon (38, 85–87). These studies and others strongly suggested the presence of an AFP cell surface receptor that constitutes an integral part of the cell membrane (88–93). Overall, the expression of AFP receptors reflected the acquisition and/or presence of phenotypic properties specific to immature, incompletely differentiated cells.

The AFP Cell-Surface Receptor

A study demonstrating the incorporation of AFP by MCF-7 human breast cancer cells was reported in 1984 (90); later that year, the same research group presented evidence for the existence of a specific membrane receptor for AFP on the surface of MCF-7 cells (91). Scatchard binding analysis of the breast cancer cell receptor revealed the presence of at least two high-affinity binding sites, with K_D of 10^{-8} and 10^{-9} M, and $N = 2{,}000$ and 135,000 sites per cell, respectively. A further study demonstrating the incorporation of AFP by human monocytes was reported in 1992 (92). Subsequent studies in using human monocytes, β-lymphoma cells, and T-leukemic cells confirmed the findings of the earlier binding studies and revealed the presence of two to three specific binding sites, with K_D values ranging from 10^{-6} to 10^{-10} M, and numerous binding sites per cells (88, 89, 91, 92). Furthermore, the receptors detected on fetal, lymphoid, and tumor cells could be distinguished from the AFP and ALB asialo-receptors, previously described on the surface of vascular endothelial cells, that are involved primarily with blood clearance activities (93).

The AFP receptor was first isolated and characterized as a specific cell-surface receptor on human monocytes in 1992 (92). After that, a human breast tumor membrane receptor that bound AFP was reported (94). This latter study described monoclonal antibodies to a human mammary

adenocarcinoma membrane receptor that inhibited AFP binding to cell membranes; together these studies aided in the isolation of 62- and 67-kDa PAS-reactive receptor components linked with a higher-molecular-mass (~200 kDa) molecular entity (94, 95). In 1997, a study confirmed the presence of a heterotrimeric protein complex composed of 65-, 130-, and 185-kDa molecules, yielding a multimer complex with a total molecular mass of 250–300 kDa (96). The trimeric protein complex was glycosylated, exhibited a high carbohydrate-to-protein ratio, and was susceptible to disulfide-bond cleavage. The trimeric complex, comprised of three noncovalently bound subunits, bound AFP (and to lesser extent, ALB) with a K_D of 2–4 $\times$ 10^{-10} *M*. The complex could be histochemically localized on the surfaces of cells from both embryonal and tumor tissues (breast, hepatoma), as had been reported in previous studies (79, 86, 89, 97). Thus, uptake and binding analyses of the AFP receptor provided the basis for a concept of an autocrine AFP/AFP-receptor system described in human monocytes, quiescent T-lymphocytes, activated T-lymphocytes, and activated T-lymphocyte systems (84, 92, 97). This autocrine loop of AFP-growth stimulation was further confirmed in tumor cell lines and was found to be intimately linked to the cell endocytotic delivery of ligands, notably fatty acids (98).

AFP: Fatty Acid Binding During Development

The presence of fatty acids on HAFP was first reported in 1978, enumerating six fatty acids: palmitic, stearic, oleic, linoleic, arachidonic (AA), and docosahexaenoic (DHA) acids (99, 100). At that same time, the binding to fatty acids and tryptophan to AFP from fetal pigs was reported in a study by Scandinavian investigators (101). Similar results were found with BAFP, which also bound fatty acids (102). During the study of binding to estrogens, it was revealed that fatty acids in serum were strong competitors of estradiol binding to RAFP (103). The fatty acid levels of RAFP derived from fetuses and pregnancy and hepatoma sera showed that AA and DHA constituted 33% of the total bound fatty acids; the highest levels were found in fetal sera (104). These polyunsaturated fatty acids (PUFAs) displayed a higher association constant (K_A = 10^{-6} *M*) than did the saturated (K_A = 10^{-4}) compounds (105); RAFP was shown to possess one high-affinity binding site and multiple (12, 13) low-affinity sites for PUFA, while HAFP presented only three binding sites, all of equivalent affinity (106–109). Similar patterns of fatty acid binding were found in AFPs of pig, bovine, rat, and human origin (101, 106, 110–113).

During development in the rat, the major fatty acids bound to AFP were DHA and AA, as assayed either from fetal serum or whole fetuses; palmitic and oleic acids were mainly bound by ALB (108, 114). Amniotic fluid AFP contained fewer fatty acids (0.8 mol/mol protein) than did fetal SAFP (1.4 mol/mol protein); especially noticeable was a reduced amount of DHA (107, 108). Levels of serum DHA bound to AFP decreased quickly after birth to a minimum at 8–10 days, which represents a period of maximal accumulation of DHA by the brain and breast-derived colostrums, accompanied by a decreased uptake by liver. It was further reported that adult rat ovarian extracts inhibited the binding of estrogen to RAFP (115). The active component of ovarian extracts, AA, also served as a strong inhibitor of E_2 binding. Since AA is a direct precursor of the prostaglandins, AFP was investigated as a regulator of prostaglandin metabolism and synthesis. Interestingly, the prostaglandins showed no affinity for binding to the AFP molecule; however, it later was demonstrated that both prostaglandins and lipoxygenase product formation were reduced when cells were maintained in the presence of AFP (115). Finally, it was shown that, when AFP was used as a protein carrier, the amount of hexaene fatty acid derivatives of linoleic acid recovered in hepatocytes was reduced by up to 50% (109, 111). This effect was explained by an efflux of hexaene derivatives from cells, with AFP as the causative agent.

Previous studies have shown that AFP also plays a role in the intracellular delivery of PUFAs into developing cells. Labeled AFP was found to enter the cells *via* coated pits and receptosomes and to move to tubular elements of the transreticular portion of the Golgi apparatus (105, 109). Fatty acids bound to AFP are transferred into cells within 5 mins at 37°C, and, following fatty acid release, AFP can be recycled back across the cell surface. Data revealed that the fatty acids (i.e., AA and DHA) bound to AFP were mainly incorporated into cell phospholipids and that 25%–40% of the incorporated AFP was secreted and released undegraded after 60 mins of incubation. The AFP first binds to an AFP cell-surface receptor, and then the fatty acid is endocytosed and transferred within the cell by a specific fatty acid–binding protein. The AFP-mediated uptake of fatty acids has also been demonstrated in human T-lymphocytes and in cancer cells (112, 113, 116).

Human AFP has been reported to bind long-chain polyunsaturated fatty acids (AA and DHA) with high-affinity $K_A 10^8$ *M*, $n \geq$ 1–3 (103, 104, 110, 116); RAFP also binds long-chain polyunsaturated fatty acids, with one high-affinity binding site and 10–12 low-affinity sites per molecule (103, 107, 114, 117). The E_2-binding sites on RAFP are subject to competition inhibition by oleic and linoleic acids and particularly by AA and DHA. These latter two fatty acids show higher levels of binding to fetal SAFP than to maternal on hepatoma AFP. Thus, it is conceivable that the binding sites for E_2 and fatty acids overlap (107). Ligand binding to MAFP has also been studied in detail by many investigators particularly in regard to estrogenic steroids and fatty acids (118–120); E_2, AA and diethylstilbestrol (DES) all bind to MAFP with decreasing affinities, respectively, K_A 0.8 $\times$ 10^8 M^{-1} (N = 0.3), K_A 0.3 $\times$ 10^7 M^{-1} (N = 4–5), and K_A 0.2 $\times$ 10^7 M^{-1} (N = 0.7; Ref. 15), and MAFP preferentially binds long-chain fatty acids (C22:4 and C22:6) that serve as efficient inhibitors of

both E_2 and DES. Murine ALB, in contrast, binds virtually no estrogen and shows a higher affinity for AA.

During pregnancy and early infancy, the biological role of HAFP in binding and trafficking of PUFAs is now well established (117, 121–123). Human AFP is known to both regulate and facilitate the entry of fatty acids (especially AA and DHA) into cells undergoing growth and differentiation (102, 112, 113). Human AFP was found to bind 16%–42% of the DHA in total fatty acid content, whereas fetal ALB bound only 4% (100, 102, 104). Human AFP reversibly binds DHA with high affinity ($K_A = 2 \times 10^7\ M^{-1}$) and transports the fatty acid mainly during the fetal, perinatal, and neonatal periods (89, 96, 102, 112). Human AFP itself undergoes transplacental passage to the maternal circulation and tissues (117, 123). Mammalian AFP *in vitro* in fetal hepatocytes has also been shown to enhance the intracellular conversion of saturated to unsaturated fatty acids (110, 118, 123).

The precise binding location on the AFP molecule is known only for a few of its ligands. For example, one major fatty acid–binding site for long-chain fatty acids has been documented to lie between residues 210 and 227 on HAFP Domain 2 (113). Lysine residues, especially Lys-223, appears to be essential for the fatty acid binding at this site. Studies employing Scatchard binding/saturation analysis have previously demonstrated that at least three potential ALB binding sites ($K_A = 10^{-7}\ M^{-1}$, $N = 3$) exist for the polyunsaturated fatty acids (i.e., AA and DHA; Refs. 106, 120). If AFP is similar to ALB in this regard, then most probably one major fatty acid–binding site exists on each of the three domains. The remaining two fatty acid–binding locations are speculated on the basis of Genbank-derived amino acid comparisons to fatty acid–related proteins. One such example of a potential HAFP Domain 1 site, residing at residues 36–69, shows an amino acid homology to fatty acid synthetase (38% identity, 32 amino acids). Indeed, when Domain 1 residues 40–60 on HAFP are compared for sequence/identity matching to the documented fatty acid–binding site on Domain 2, several interesting points emerge (Table 2). First, it can be seen that each of the 20 amino acid sequence stretches contains three or more lysines, which are essential for fatty acid binding. Second, one or more lysines are located at the amino-terminal side of the sequence amino acid stretch. Third, the crucial amino acid for complexing to the carboxy group of the bound fatty acid has been identified as the lysine (see asterisk) positioned 13–14 amino acids from the amino-terminal lysine. These three criteria from Domain 2 fit the amino acid sequences on both Domain 1 (residues 41–62) and Domain 3 (residues 418–437), compared to Domain 2 (residues 208–227). Both amino acid sequences stretches on Domains 1 and 2 demonstrate 60%–65% identity/similarity matching to the documented fatty acid–binding site on residues 208–227 of the second domain. The third AFP fatty acid–binding site, which resides on Domain 3, apparently overlaps or lies directly adjacent to the documented estrogen-binding site on RAFP, according to previous competitive-binding reports (75, 168; Table 2, part B).

Binding, spectral, and immunological studies have demonstrated that conformational changes in the tertiary structures of rodent and human AFPs can be induced by a high free fatty acid (PUFA) environment (121). In contrast to the PUFAs, saturated fatty acids had no effect on the tertiary properties of all AFP species tested. Human AFP measured by RIA/ELISA in fetal, hepatoma, and cord serum showed reductions in AFP levels of 80%, 50%, and 5%, respectively, in these fluids, following the change in tertiary structure. Furthermore, a transient rise in plasma PUFA levels led to a loss of AFP immunoreactivity in 21- and 28-day-old rats (122). In all cases, the fatty acids induced a rapid and reversible conformational change in the RAFP molecule. At the placental interface, PUFAs were found at low levels in maternal blood but at high levels in intervillous/umbilical blood vessels; the AA and DHA concentrations were also highest here (117, 123). The conformational state of AFP was found to differ in the intervillous spaces, suggesting that AFP was heavily loaded with PUFAs at the feto-maternal interface (123). High concentrations of PUFAs stimulated E_2 binding and inhibited progesterone (PG) binding, suggesting that PUFAs modulate the steroid hormone message by amplifying the E_2 signal and damping the PG signal (119).

Interactions of AFP with Estrogen During Development

Although HAFP was initially reported to bind few, if any, estrogenic steroids (124), additional evidence now indicates that HAFP (obtained by butanol extraction of fetal tissues) can bind to immobilized estrogen columns, suggesting the existence of a partially occluded binding site (125). Previous data had implied that <1.0% of the HAFP population was capable of such binding (126, 127) and that the butanol either induced a conformational change or removed a bound ligand that normally interferes with the estrogen binding site (127). This is because AFP is able to bind alcohols; furthermore, HAFP was found to bind longer-chain alcohols more strongly than it did low-carbon chain alcohols (butanol > propanol > ethanol > methanol; Ref. 128). The reported alcohol- and drug-binding affinities of HAFP displayed a K_A of $10^6\ M^{-1}$ and binding site numbers 1.2–3.4 (129, 130). Thus, HAFP is capable of binding butanol, which may have outcompeted a bound agent to which HAFP had been complexed. The use of purified RAFP also has provided a means by which to study the *in vitro* binding and *in vivo* transport of steroids by this fetal protein (131–135). In a study employing RAFP fractions on lectin columns, low-carbohydrate forms bound E_2 with high affinity ($K_A = 10^8\ M$) and 0.5 sites per mole of protein (131). It has been demonstrated that RAFP binds estrone (2.74×10^7) with higher affinity than it does E_2 ($K_A = 1.83 \times 10^7\ M^{-1}$; Refs. 137–140), contrast to the behavior

Table 2. Proposed and Documented Amino Acid Sequences for Fatty Acid– and Estrogen-Binding Sites on the Human Alpha-Fetoprotein (HAFP) Molecule[a]

Part A: Fatty acid–binding sites

		*														*				
HAFP 41–60	Y	K	E	V	S	K	M	V	K	D	A	L	T	A	I	K	P	T	G	D	Domain 1
HAFP 208–227	M	K	N	F	G	T	R	T	F	Q	A	I	T	V	T	K	L	S	Q	K	Domain 2
HAFP 418–437	K	K	A	P	Q	L	T	S	S	E	L	M	A	I	T	K	R	M	A	A	Domain 3

HAFP 208–227 is a documented fatty acid–binding site, while HAFP 41–60 and HAFP 418–437 are proposed sites based on sequence identify/similarities matches. Asterisks indicate essential lysines. Note the overlap of the fatty acid–binding site 418–437 and the estrogen-binding site 427–438 (see below).

Part B: Estrogen (estradiol)-binding sites (Domain 3)

Site 1: Primary, high-affinity (10^{-8} *M*) binding site

									*			*	*	*		
HER 418–432	E/G	M	V	E	I	—	F	D	M	L	—	L	A	T/S	S	Type of site documented
RAFP 419–433	E	L	I	D	L	T	G	K	M	V	S	I	A	S	T	Documented
MAFP 422–435	E	L	I	D	L	T	G	K	M	V	S	I	A	S	T	Proposed
HAFP 427–438	E	L	M	A	I	T	R	K	M	A	A	T	A	A	T	Proposed
Consensus	E	L	I	D	L/I	T	G	K	M	V	S	I	A	S	T	

Site II: Secondary, low-affinity (10^{-5} *M*) binding site

	*	*		*			*					*				
HER 504–517	L	A	Q	L	L	L	I	—	S/L	H	I	R	H	—	M	Type of site documented
RAFP 450–464	L	A	D	I	Y	I	G	H	L	C	L	R	H	E	A	Proposed
MAFP 452–466	M	A	D	I	I	I	G	H	L	C	I	R	N	E	A	Proposed
HAFP 454–468	A	A	D	I	I	I	G	H	L	C	I	R	H	E	M	Documented
Consensus	L	A	D	I	I	I	G	H	L	C	I	R	H	E	A	

[a] HER, human estrogen receptor; RAFP, rat alpha-fetoprotein; MAFP, mouse alpha-fetoprotein; numbers indicate amino acid sequence numbers of the protein; data extracted from the following references: Part A, Refs. 116, 117, 126, 127; Part B, Refs. 131, 132, 152, 154. Sequence matches performed as described in Ref. 176. Asterisks signify key residues for binding.

of MAFP (see below); MAFP binds with high affinity to E_2 ($K_A = 0.4 \times 10^8\ M^{-1}$, $N = 0.3$; Refs. 131, 134, 135). The carbohydrate (CHO) prosthetic group of MAFP plays a significant role in E_2 binding, in that a fraction devoid of CHO groups results in the appearance of very high affinity binding sites (~$10^9\ M^{-1}$). As expected, mouse embryonic serum has a very high affinity for both (E_1) and E_2, peaking at 15–18 days of gestation, although little E_1 is initially bound at Day 12 (135). In fact, the major estrogen-binding component in mouse amniotic fluid is AFP (136), and it serves as a secondary E_2-binding agent in rodent uterine tissues (137). Further serum elevation of MAFP in adult mice can be induced by intraperitoneal injections of either estriol or E_2 (100–1,000-μg quantities; Refs. 138, 139). Because MAFP binds with such avidity to both E_2 and E_1, estrogen-based affinity chromatography has served as a useful means by which to purify the protein (126, 127).

Physiological studies on AFP-estrogen interaction have been undertaken in a variety of animal models; RAFP was shown to inhibit the formation of water-soluble metabolites of E_2 and E_1, when microsomes from rat liver were incubated in the presence of NADPH, and to regulate the activity of 17β-hydroxy steroid dehydrogenase *in vitro* (140). Alpha-fetoprotein has also been associated with the delay in the onset of puberty in postnatal rat pups (141). Finally, the injection of AFP during the prepubertal period in rats has resulted in a decreased number of primordial and primary follicles in the ovary (142). The injected RAFP was localized to the zona pellucida of follicles undergoing atresia.

The estrogen-binding interface on rodent AFP has been reported to occupy a region between residues 423 and 444, forming an α-helical segment that lies adjacent to a potential β-sheet/turn structure extending from residues 445–480 (143, 144; Table 2). The former site (residues 423–444) represents a major hydrophobic binding pocket on HAFP that binds few estrogenic steroids, in contrast to rodents (144, 145). In humans, both regions (residues 423–444 and 445–480) display overlapping binding sites for fatty acids, DES, protease inhibitors/substrates, retinoids, warfarin, coumarin, phenylbutazone, pyrazolic drugs, and anthranilic acid (128, 129, 145). Previous competitive-binding studies employing RAFP had already determined that estrogen was bound on RAFP Domain 3, together with retinoids and fatty acids (146, 147).

Both RAFP and MAFP avidly bind estrogens (E_2, E_1)

with high K_d values (10^{-8} M^{-1}); however, HAFP binds few of these estrogens, as discussed above. One high-affinity region (Site I) and one low-affinity region (Site II) have been reported for AFP (147). Through the use of recombinant technology involving human/rat hybrids, the primary estrogen binding Site I in RAFP has been determined to lie between residues 419 and 436, corresponding to HAFP residues 424–439 (Table 2). The five amino acids crucial to the binding on RAFP are glycine-425, methionine-427, isoleucine-430, alanine-432, and threonine-432 (144). These amino acids match precisely to the MAFP residues glycine-428, methionine-430, isoleucine-433, alanine-434, and threonine-435. In contrast, three of five of these amino acids in HAFP, a protein that binds only scant amounts of estrogen, show substitutions, namely, glycine → arginine, isoleucine → threonine, and serine → alanine. Interestingly, 8 of the 15 amino acids in this HAFP sequence stretch display substitutions, when compared to the rodent AFPs. Moreover, when the amino acid sequences of human and rodent AFPs are compared to an estrogen-binding region on the human estrogen receptor (HER; residues 419–431), it can be seen that four of the five amino acids crucial for estrogen binding to rodent AFP are matched and aligned to those of HER. As displayed in Table 2, the remaining hydrophobic amino acids in human and rodent AFPs are also present in HER, namely, leucine and valine. It is obvious that in the HAFP molecule, multiple (five) alanines have replaced many of the crucial amino acids present in both RAFP and HER. However, a single alanine that is retained in all species is probably significant. Thus, this 15-amino acid region on the various AFPs seems to represent the high-affinity binding site for rodent and to a lesser extent human AFP.

For the second AFP estrogen-binding region, Site II, a different scenario emerges (Table 2). This segment has been shown to bind E_2, through the use of AFP-derived peptides, and it probably serves as a secondary, lower-affinity binding site in both HAFP and rodent AFPs. As seen above, HAFP appears to largely lack crucial amino acids in the primary binding site, but it has retained most of these residues in the secondary binding site. The latter, secondary site is highly hydrophobic (leucines, isoleucines, alanines) in all three AFP species (HAFP, RAFP, MAFP) and in HER (five leucines, two isoleucines, one alanine). The various AFP molecules display a common cysteine, unlike HER, but otherwise they show similar arginine and histidine positionings. In both the AFPs and HER, the composition and placement (position) of leucines, isoleucines, arginines, and histidine as the dominant amino acids are shared. The HER segment is devoid of glycines and cysteine in its secondary binding site, in contrast to the rodent and human AFPs. Thus, the lower binding affinity of estrogen-binding Site II (10^{-5} M^{-1}) as compared to Site I (10^{-8} M^{-1}) may be ascribed to the predominant leucine/isoleucine hydrophobic composition of Site II since Site I contains mostly methionine, threonine/serine, and alanine. Cysteine does not seem to be required for estrogen binding at this secondary site. In the native state of the HAFP molecule, these cysteines are disulfide bonded. While AFP Site I is primarily committed to estrogen binding/ligation, Site II is also thought to serve as a docking site for proteins of the heat-shock protein (HSP) family, such as HSP-70, and HSP-90, as in HER (148). Such docking sites for the HSPs are also known to be involved in protein folding/unfolding activities (149).

AFP and Insulin/Carbohydrate Chemistry

Insulin is the most potent anabolic hormone known, promoting synthesis and storage of carbohydrates, lipids, and proteins (150). The interaction of insulin with AFP in developing mammals has previously been described (151); however, effects of mammalian AFP in the chick embryo have not been reported until now. In pregnant mice, a single pulse of insulin at Day 13 of gestation produced elevated maternal SAFP levels and increases fetal mass, while long-term treatment with insulin yielded lower levels of maternal SAFP and lower fetal mass per animal (152). Human AFP has been employed in the clinical laboratory as a gestational age–dependent fetal defect marker and is presently utilized as a screening agent for neural tube defects (153) and aneuploidies (154). The neural tube defect screening procedure identifies a distinct subpopulation of women who exhibit insulin-dependent diabetes during pregnancy. These pregnant women display maternal SAFP concentrations that are 20% lower than those of age-matched controls (153). In avian models of insulin teratogenicity, there is seen a form of caudal vertebrae rumplessness in early development that resembles the mammalian syndrome of neural tube defects (155–158). Closure defects of the neural tube in chick fetuses are accompanied by AFP levels 4-fold higher than in controls. Insulin exposure in the later development of the chick fetus gives rise to a malformation syndrome, classified as achondroplasia, involving micromelia, a shortening of the skeletal limbs (159). Both the early and later fetal chick malformations are associated with fetal demise. Paradoxically, insulin at physiological doses shows growth-stimulating effects, while high doses result in growth disruption.

Purified HAFP has been found to contain 4.5% carbohydrate and to possess 2–6 isoforms due to its carbohydrate heterogeneity. The sugars present on the AFP molecule include N-acetylglucosamine, mannose, galactose, sialic acid, and small amounts of glucose (160). Interestingly, glucose was found to co-purify with HAFP even after rigorous purification schemes and strategies had been followed (161). Then, in 1981, a direct relationship of glucose levels with amniotic fluid AFP levels was reported for the 16–22-week human gestational period (162). However, it was not until the late 1990s that studies of AFP-derived peptide fragments from the amino-terminal side of AFP Domain 1 were found to have glucose/insulin-related activities. A synthetic peptide, duplicating amino

Table 3. Genbank Amino Acid Sequence Matching of the Alpha-Fetoprotein–Derived Peptide (amino acids 445–480) with Conserved Sequences from Various Insulin/Glucose-Related Proteins and Transcription Factors.[a]

		% ID/SIM	% Total
1. Glucose/insulin shock			
HAFP 445	LSEDKLLACGEGAADIIIGHLCIRHEMTPVNPGV	100/100	100
Mus IGF-II rec.	EARLLAC RMTPLTPPL	56/25	81
Pan vector protein	LSENRLL	71/28	99
Mus IGF-BP	LLPCAHGAA	66/11	77
Pan reth acid Glu-P	KCGSSNPQLY--RLCIRR	40/43	83
Hum Na^+/Glu-Ct	LSGHVLRSCIHPAGSXGLEHLCLRH	40/20	60
Rat Pan Ass. Prot	LTFGEVCTRH	40/20	60
DRo Br-7-Less	SIVGHLCERI	60/10	70
Hum Glu-Pyro	RLFGHLCIRX	60/20	80
Pig Na/Glu-CT	IILSQLCIFL	50/20	70
Pig growth hormone	GEQLCLLVAGHCCPSPVPPLNPGK	42/17	59
Rattine SomatR	RTTPIAPGV	50/20	70
Human SrctyK	EMAPIWPGA	50/20	70
2.Transcription/growth factors			
Human HGMP	LSEDKLLAVARE	66/8	74
Ovine c-Myc	LSEARVVGCGEG	58/33	91
Human Zn-Fg	LSDHKLLESTCK	42/25	67
Human HGMP	VAQIIIGHLCIR	83/7	90
Human Zn Fg	AGGTLVGHLCVR	50/25	75
Yeast NRTF	LRDIIIGSERLK	50/20	70
Yeast forkhead	CLLIIYAHLCIX	60/10	70
Dros. AP-1	MLIIIFTNFCIR	42/25	67
Human PAX-3/forkhead	IMTPVDPGV	70/10	80
Human forkhead	EMTPVDPGV	60/10	70
Human TF11D	PMTPATPGS	50/20	70
Human Src-Tyc	EMAPIWPGA	50/20	70
Human Kid-TS	KSTGANPGV	50/10	60
Human Cad-TS	EMTPVLEAI	50/0	50
Human FTZ-F1	KPTPISPGY	40/30	70

[a] AF-1, Fos/Jun oncogene protein complex; BP, binding protein; Cad-TS, cadherin tumor suppressor; C-myc, cellular oncogene; Dro Br-7-Less, Drosphilia bride of 7-less; FTZ-F1, AFP transcription factor; Glu-Pyro, glucose pyrophosphate; HGMP, high group mobility protein; IGF, insulin growth factor; Kid-TS, Wilm's kidney tumor suppressor; Mus, mouse; Na/Glu-Ct, sodium/glucose co-transporter; NRTF, negative regulating transcription factor; Pan reth Acid Glu-P, plant acid glucophosphatase; Pan vector, pancreas vector protein; SomatR, somatostatin receptor; SrctyK, SRC-domain tyrosine kinase; TF11F, transcription initiation factor (Tata-Box); TS, transcription factor; Zn-Fg, zinc finger protein.

acids 13–19 (LDSYQC) of HAFP Domain 1, was reported to influence glucose uptake by human red blood cells in the clinical laboratory (163). The LDSYQC peptide was found to stimulate the entry of glucose into red blood cells from insulin-dependent diabetic (IDD) children after a 1.0-h treatment in 10^{-8} *M*–10^{-6} *M* peptide concentrations *in vitro* (164). It is of interest that an amino acid sequence on the insulin α-chain (residues 17–21) has been found to be structurally homologous to the AFP-derived LDSYQC sequence. Sequence matching to HAFP amino acids 13–19 has also shown identity/similarity to epidermal growth factor (amino acids 36–32) and glycodelin (amino acids 67–73 and 114–120). Finally, it was shown that a peptide fragment derived from HAFP (amino acids 445–480) is capable of reducing both fetal death and malformations in chick embryos subjected to high doses of insulin during early gestation. The AFP-derived peptide reduced fetal death by up to 73%, and fetal anomalies were diminished by 50%, at the cost of growth restriction in the fetuses (165).

It is of further interest that insulin signaling regulates not only metabolic and developmental pathways but also life span. Exposure to insulin has been proposed as a major factor in the regulation of the rate of aging in both mammals (166) and lower animals (167). The latter studies have provided evidence that the insulin/insulin growth factor signaling system is related to the regulation of aging and life span in insects and worms (168, 169). In mammals, the overexpression of high levels of growth hormone (GH) in transgenic rodents also results in an impairment of insulin signaling and is associated with a reduction of rodent life span (170). In nematodes, the DAF-16 and DAF-2 proteins serve to mediate and integrate developmental/environmental inputs in the aging *C. elegans* and in the duration of the dauer larval stage. The human orthologs of DAF-16 and DAF-2 are the FKHRL1 forkhead transcription factor (TF) and the insulin/IGF type II receptor (Table 3; Ref. 180). The FKHR-TF is induced by glucose starvation and oxidative stress and activates AMP-induced protein kinase-β (169).

Both human proteins exhibit amino acid sequence identity to the P149 AFP peptide molecule (IGF receptor; Ref. 165). In a non–species-specific manner, the P149 peptide has previously been shown to inhibit frog metamorphosis by maintaining the tadpole in its larval form (171) and to extend the prepubertal period by suppressing E_2-induced uterine growth (165). High levels of AFP, if sustained, seem to prolong and favor maintenance of the larval (juville) form of the organism, thus extending life span (15).

The FKHR (FOXO-1)-TF protein is a key regulator of glucose homeostasis, cell-cycle progression, and apoptosis (Table 3; Ref. 180). Some cells are programmed for apoptosis during embryonic life and exhibit very short life spans during embryogenesis, histogenesis, metamorphosis, and endocrine-dependent tissue atrophy. Throughout development, and especially in the early embryo, a competition exists between cell death signals and survival (rescue) signals, such as in gastrulation and in cavitation of the blastula. Such FKHR-TF–regulated apoptosis is instrumental in creating lumina in a variety of embryonic structures (16). Previous studies have shown that AFP is able to induce apoptosis in tumor cells through activation of caspase-3, bypassing FAS dependent and tumor necrosis factor receptor–dependent processes (19). Recent studies by Semenhova and co-workers (19), using a cell-free system, have shed further light on this regulation of apoptosis. These investigators showed that AFP mediated the processing and activation of caspase-3 and caspase-9 by a synergistic enhancement of low-dose cytochrome C–mediated signals. This group further demonstrated that AFP positively regulated cytochrome c/ATP–mediated apoptosome complex formation, enhanced recruitment of caspases for the complex, and stimulated release of caspase-3 and caspase-9 from the apoptosome. Their data suggested that AFP regulates cell death by displacing apoptotic inhibitors from the apoptosome, resulting in activation of caspase-3 and release of it from the complex. These investigators further proposed that AFP was a feto-embryonic defense system with the purpose of preventing malignant transformation of developing cells since AFP targets only neoplastic and activated immune cells (19). However, FKHR-TF regulation of cell death could also be intrinsic to regulation of life span and longevity of juvenile organisms *via* its modulation of apoptosis during the onset of puberty.

AFP, Thyroid Hormone, and Hypothyroidism

In developing and neoplastic cell cultures, AFP and the thyroid hormones display an inverse relationship in that cells exposed to increasing concentrations of triidothyronine (T_3) show decreased secretion of measurable AFP (172–174). *In vivo* studies have demonstrated that thyroxine (T_4)-treated newborn rodents display depressed SAFP levels that were attributed to blockage of hepatic AFP synthesis (174). In human newborn studies, a reciprocal relationship between serum AFP levels and congenital hypothyroidism also exists (175). Furthermore, native rodent AFP binds T_4 (176). Thus, AFP and thyroid hormone levels have consistently displayed an inverse relationship in both *in vitro* and *in vivo* model systems.

Studies have also documented a relationship between SAFP and congenital hypothyroidism (CH) that paralleled a correlation between elevated SAFP and elevated thyroid-stimulating hormone (TSH)/low T_4 in postnatal life (177–180). Thus, it is salient that purified TSH was reported to form a molecular complex with purified HAFP *in vitro*, suggesting protein-to-protein interaction, at least at high serum concentrations of these analytes (Fig. 1; Refs. 178, 179). In a gel filtration chromatography study, gel retardation occurred, thus demonstrating that AFP complexed with radiolabeled TSH and eluted earlier than did free ^{125}I-TSH. In the same report, heterocomplexing of the TSH and AFP macromolecules was also demonstrated in polyacrylamide gels following electrophoresis. However, the aggregation of the two protein molecules has yet to be explained other than simple physical binding forces such as hydrogen bonding and Van der Waal's forces. Overall, elevated levels of AFP were found to be consistent with elevated levels of TSH, and the elevated AFP levels in CH can readily be distinguished from those in premature infants through ancillary thyroid assays.

The physiological mechanism by which SAFP is elevated postnatally in CH remains to be clarified. Larsson and co-workers (172) suggested that either the rate of AFP synthesis in liver is increased, or else the repression of this synthesis is delayed in the hypothyroid fetus/infant. However, Mengreli and co-workers (181) argued that the half-life of AFP is extended in these infants (12 days vs. the normal 5 days) and postulated that low T_4 levels could be responsible for a slower catabolism of SAFP in the liver. In a report predating these proposals, Belanger and co-workers (174) had demonstrated that both glucocorticoid and T_4-treated newborn rodents displayed depressed SAFP levels as a result of selective blockage of AFPs hepatic synthesis rather than as the result of altered serum clearance of the fetal protein. In addition, thyroidectomy of the rodents did not modify the effects of administered glucocorticoid or T_4. The aforementioned studies and the clinical observations discussed above lend credence to the proposal that either low T_4, elevated TSH, or both coexist with elevated AFP levels in the neonatal period.

Fetal Erythropoiesis

The existence of a relationship between AFP and fetal erythropoiesis comes as no surprise since the fetal liver and yolk sac constitute the major embryonic/fetal sites of both AFP synthesis and erythropoiesis (182). In fact, tumors of the yolk sac and the liver (hepatomas) are known to synthesize and secrete AFP (183). Patients with hepatomas are known to present with erythrocytosis and elevated erthyropoitein, which are associated with polycythemia in

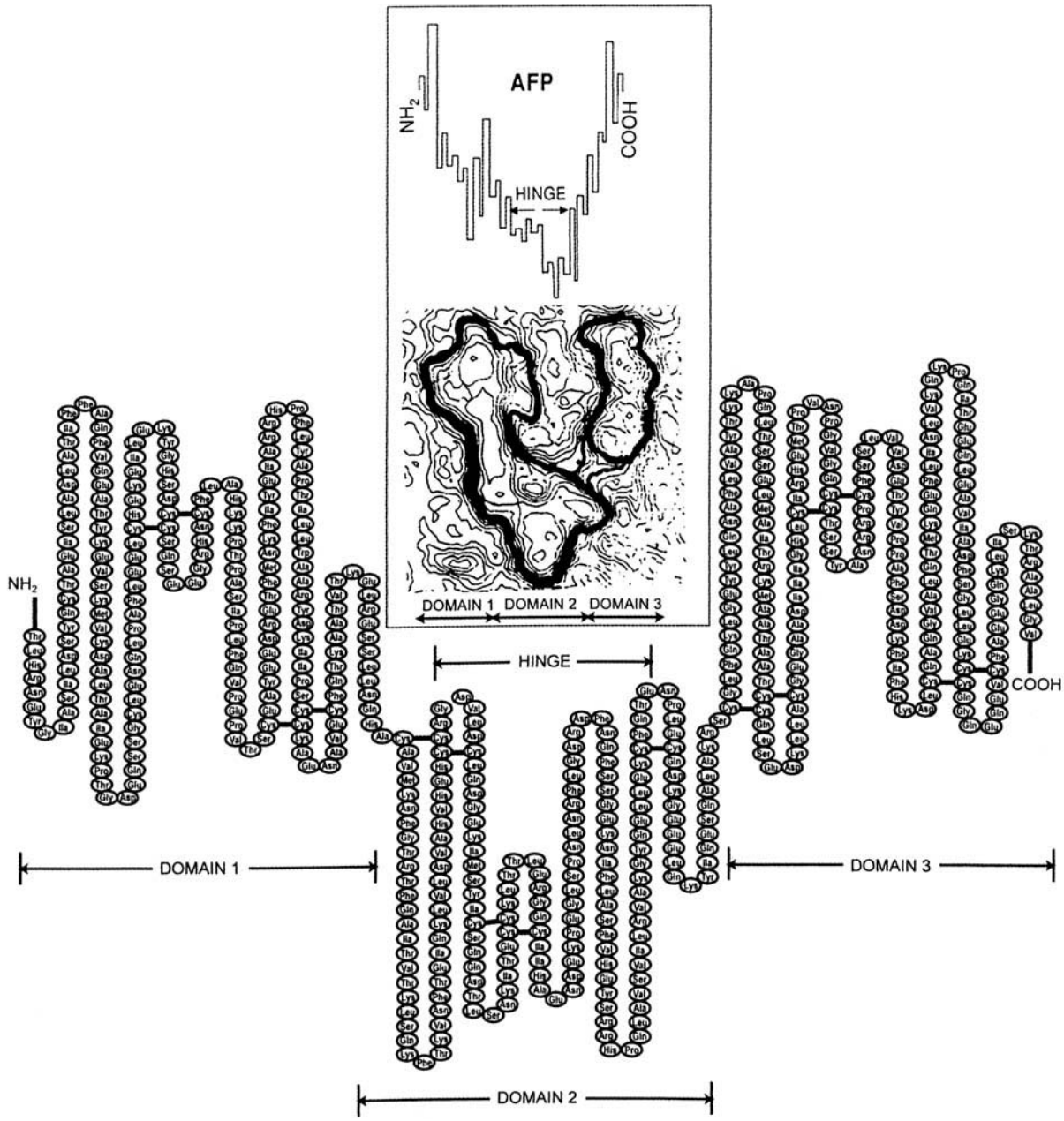

Figure 1. The primary and secondary structure amino acid sequence of human alpha-fetoprotein (AFP) is displayed. Note that the HAFP molecule is composed of three domains in a U-shaped configuration as confirmed by electron dot contour mass mapping. Human AFP belongs to the albuminoid gene family, which is characterized structurally by cysteine residues that are folded into layers that form loops dictated by disulfide bridging (15). The third domain is positioned close to a proposed "hinge" region (see arrows). The hinge concept developed from the observation that HAFP has two disulfide bridges fewer than does human albumin, providing it with a means of molecular flexibility (82). The panel in the top inset displays the two-dimensional aspect of the AFP domain structure; the panel in the bottom inset shows the electron dot contour map of the human AFP molecule (see Refs. 16, 17).

these individuals (184, 185). Levels of SAFP are also elevated in weanling rats born to mothers with anemia that was induced by serial bleeding episodes during pregnancy (174). Furthermore, elevated levels of serum SAFP persist in the nude mouse up to or beyond the 20th week following birth (186). This maintenance of high SAFP levels has been attributed to the continued presence of hematopoietic foci present in the livers of nude mice during much of their adult life span. Finally, high levels of SAFP are maintained in tumor-bearing mice that have verified hemolytic anemia of the patient (187). Such situations regarding AFP have been confirmed in subsequent studies of fetal asplastic crisis (189).

Meticulous clinical studies by Bartha and co-workers determined that significant negative correlations existed between maternal AFP and fetal hemoglobin levels (182, 188). These investigators also found a negative correlation between fetal levels of SAFP and levels of fetal red blood

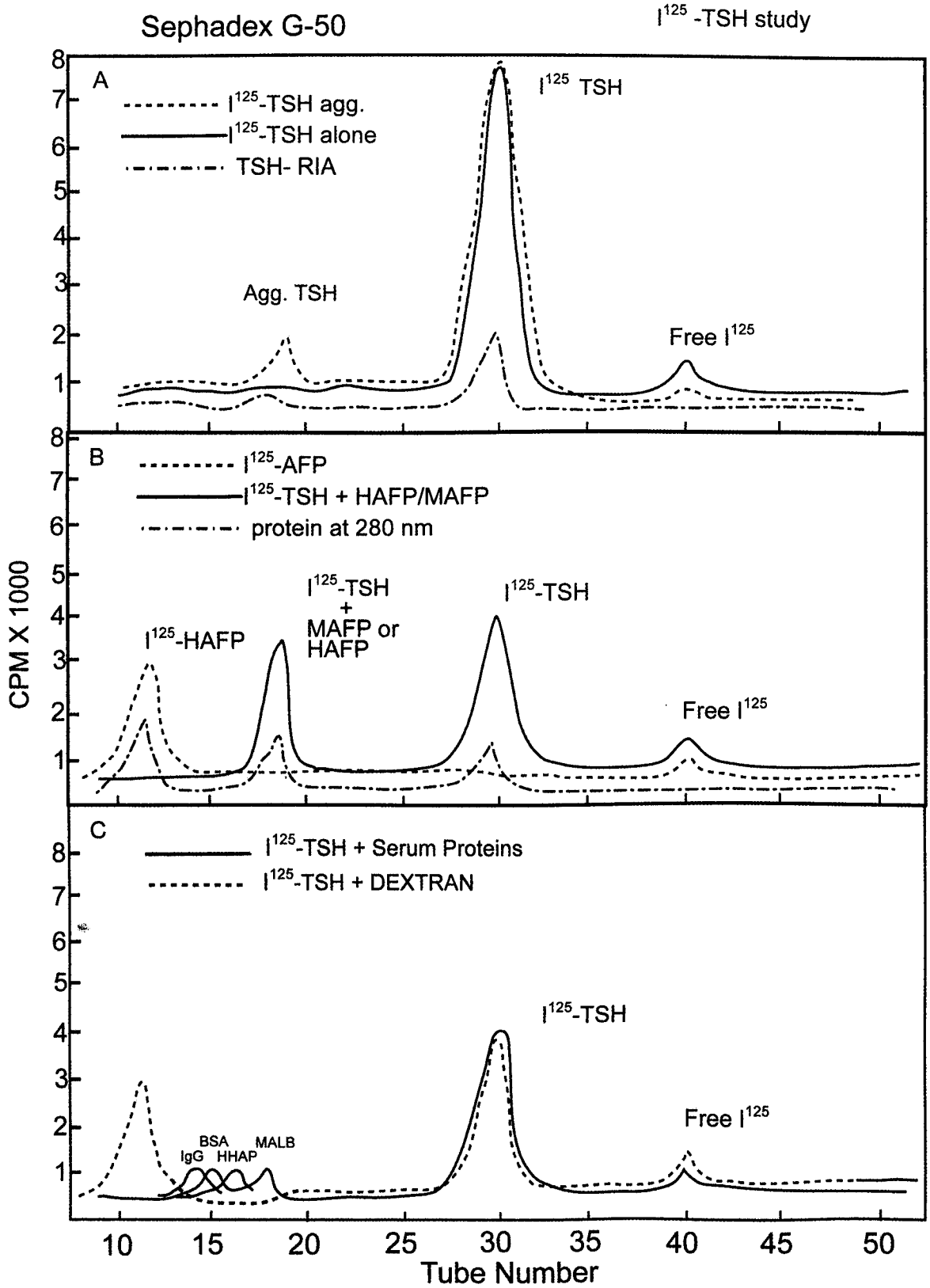

Figure 2. A chromatographic elution profile is displayed for ^{125}I-thyroid-stimulating hormone (TSH) with or without addition of protein complexing agents. Radiolabeled I^{125}-TSH was subjected to Sephadex G-50 gel filtration column chromatography using a peristaltic pump (see Refs. 178, 179). The ^{125}I-TSH eluted in the fraction tubes 28–30, while dextran (A and C) eluted with the void volume in fractions 10–12. The precise elution location of the TSH was confirmed by radio-immunoassay (RIA) of each tube fraction in panel A. Free iodine, when present, eluted at or near tube 40. While aggregated TSH eluted earlier than monomeric TSH, aggregates of TSH with AFP or control proteins were

cells, hemoglobin, hematocrit, erythropoietin, and transferrin. Concomitantly, in this study, erythropoietin levels were positively correlated with levels of fetal red blood cells, hemoglobin, and erythroblasts and with hematocrit. This research group concluded that AFP must play a regulatory/modifying role in fetal erythropoiesis. Thus, we might expect a high AFP level in anemic fetuses and a low one under conditions of hemoconcentration.

A relationship between of AFP level and serum folate deficiencies during pregnancy is also related to anemias. The increased demand for folic acids (FA) during pregnancy results from FA transport to the fetus, placental tissue growth, elevated erythrocyte synthesis, and urinary loss replacement. It is generally accepted that a steady depletion of maternal serum folate occurs during pregnancy and is accompanied by a mild physiologic anemia (190). The serum folate decline has been attributed to rapid plasma clearance, fetal transfer, hemodilution, and possibly hormonal effects, as seen in users of oral contraceptives. There appears to be an elevated incidence of the megaloblastic-type anemias in folate-deficient pregnant women (191). In addition, a number of complications have been associated with folate deficiency in pregnant women, although some of these associations are questionable. Among the complications are (i) abruption placentae; (ii) toxemia of pregnancy; (iii) spontaneous abortion and fetal death; (iv) neural tube defects, including spina bifida and anencephaly; (v) hydrocephalus; (vi) prematurity (low birth weight); (vii) hemolytic anemias; (viii) various anatomic congenital malformations; and (ix) twin pregnancies (192, 194). It is quite remarkable that AFP concentrations tend to be elevated in pregnancy complications linked to folate deficiencies. However, AFP is elevated in some instances where deficiency in FA is implicated and in which a biochemical rather than an anatomic basis is involved.

In light of the above associations, we may consider the following reported observations. First, both HAFP and ALB (193, 194) can bind FA in a low-affinity, high-capacity manner (Fig. 2). Even though FA binding affinity is low (10^{-3} M^{-1}), the high concentrations of AFP in early pregnancy (3 mg/ml) are sufficient to bind a considerable amount of FA. It is of interest that HAFP displays a Genbank amino acid sequence identity to a murine folate-binding protein at residues 352–372 (50% identity) on Domain 2, suggesting that a folic acid–binding site could reside at or near this location. Second, elevated SAFP levels have been reported in patients with severe anemias (195). Third, the normal decline of maternal serum folate appears to parallel the normal fall in HAFP concentrations in both amniotic fluid (13–40 weeks gestation) and maternal serum (30–40 weeks gestation; Ref. 194). Fourth, in pups born to nude mice, both elevated SAFP levels and liver hematopoiesis are maintained well into adulthood (186). Thus, conditions that modulate or interfere with hematologic maturation processes in the prenatal, perinatal, or postnatal period appear to influence AFP fluid concentrations. The AFP-FA relationship is deemed important, in light of the studies linking the prevention of neural tube defects with periconceptional FA supplementation (196).

Hematopoietic stem cells are derived from the mesodermal germ layer in the embryo and give rise to all known adult hematopoietic cell lineages (197). However, recent data suggest that bone marrow cells with a hematopoietic stem cell profile can also cross-differentiate (as totipotent cells) to endodermal cell types, such as liver cells (see below). Human AFP, a differentiation marker for endodermal cells, has long been thought to be tightly regulated, in a tissue-specific manner, during development (198, 199). However, in a recent report, two new HAFP mRNA transcripts were described as AFP-variant (V) forms in hematopoietic progenitor cells that are not expressed in adult cells (199). In the AFP-V, exon 1 of AFP is replaced with one or two exons in the 5′ untranslated region of the AFP gene. In cell culture lines, the AFP-V transcripts were detected in bone marrow, thymus, and brain but were not detected in spleen, intestine, or liver or in the hepatoma HepG2 cell line. Hematopoietic progenitor cells purified from cord blood (flow-cell sorting) also expressed the variant transcripts. The investigators asserted that certain hematopoietic stem cells are capable of expressing fetal protein transcripts thought to be unique to the endoderm.

Acquired Immunodeficiency Disorders (AIDS)

Although there may be many explanations for the lack of fetal infection in HIV-positive mothers in the first and second trimesters (200–206), a possible link to HAFP can be examined. A recent study has documented that the relatively low rate absence of HIV-1 fetal transmission during pregnancy is associated with the elevated maternal SAFP levels (205). The pioneering studies of Uriel and his associates in 1987–89 demonstrated specific uptake of HAFP by receptors on human T-lymphocyte blast cells during antigen-induced transformation and in malignant lymphoid cells (207, 208). In the course of these detailed studies, Uriel's group reported an impairment in the ability of AIDS (HIV) patients' peripheral blood mononuclear cells to internalize AFP (209, 210). The authors' AFP endocy-

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retarded and eluted in earlier tubes, thus confirming heterocomplex formation or interaction of the two protein moieties (Panels A and B). Protein was monitored at a wavelength of 280 nm. Radiolabeled ^{125}I-AFP alone eluted at or near the void volume of the column slightly after dextran. Note that complexing of various proteins (i.e., AFP with the radiolabeled TSH) reduced the peak height of the ^{125}I-TSH fraction. Other serum proteins tested included BSA = bovine serum albumin; MALB = mouse albumin; HHAP = human haptoglobin; and human IgG = immunoglobulin-G. None of the non-AFP proteins showed complexing activity.

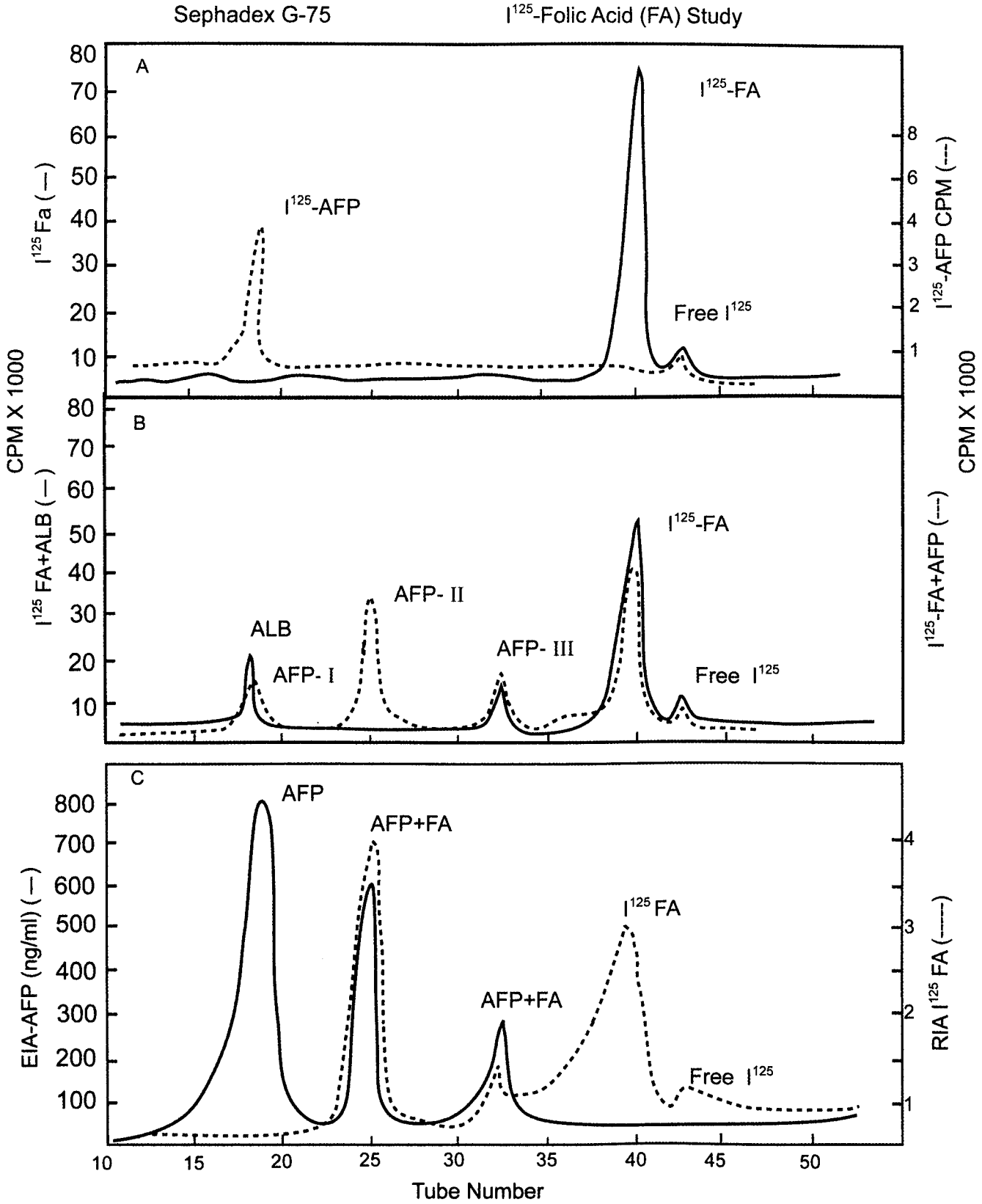

Figure 3. A chromatographic G-75 elution profile is shown for radiolabeled folic acid (FA) with or without complexing proteins (A). Radiolabeled ^{125}I-FA was subjected to Sephadex G-75 gel filtration column chromatography using a peristaltic pump (data obtained from Refs. 193, 194). The ^{125}I-FA eluted from the column in fraction tubes 38–40, while ^{125}I-FA heteroprotein complex formation was detected as gel retardation in several tube locations between tubes 15 and 33, thus indicating varying degrees of dissociation (low-affinity binding) of ^{125}I-FA bound to AFP. (B and C) Confirmation of the tube location for FA was made by RIA (B and C). Likewise, the fraction location of AFP shown in panel B was confirmed by

tosis assay clearly revealed a defective uptake of AFP in AIDS, in lymphoadenoapathy syndrome, and in mitogen-responsive T cells of asymptomatic patients. They predicted that the reduced capacity to bind and internalize AFP in early-stage nonsymptomatic HIV/AIDS patients held the potential to form the basis of a prognostic test. These investigators further reported good concordance of the defective AFP uptake with an impaired expression of IL-2 receptors on the lymphoid cells (211). Finally, these investigators noted that the seroconversion from HIV-negative to HIV-positive was accompanied by the onset of a progressive deterioration in the patients' AFP uptake capability.

Uriel's group then attempted to determine whether the AFP-uptake impairment was due to an expression of inhibited AFP receptor or to a target signal transduction defect. They studied AFP endocytosis in peripheral mononuclear cells (PMCs) from asymptomatic HIV-positive patients as well as in HIV-transfected PMCs (*in vitro*) from healthy donors in phytohemaglutinin-stimulated and nonstimulated conditions. Their results showed that defective AFP endocytosis was a consequence of an abnormal mitogenic response of PMCs associated with the presence of the HIV virus. Also, a lowered level of IL-2 receptor was found in this study (210). Thus, their results reflected both the status of T-cell activation associated with HIV infection and the PMCs' responsiveness to mitogenic stimulation. The study by Uriel and co-workers in 1994 demonstrated a considerable loss of membrane fluidity of the PMCs, as evidenced by elevated values of the cholesterol/phospholipid (CH/PL) ratio in cell membranes from AIDS patients (211). Relative to normal cells, the expression of AFP and IL-2 receptors also appeared considerably reduced in AIDS-related complex disorders and in AIDS patients. Thus, the HIV infection disrupted the fluidity of the cell membrane and altered the normal sequelae of lymphocyte antigen-activation and blast-cell transformation.

Later, in 1997, another French group reported that AFP interacted with the HIV-1 gp 120/160 viral coat proteins (212); thus, AFP inhibited the infection of primary monocyte-derived macrophages by certain HIV-1 viral strains. Serving as an inhibitor, AFP acted at the cell-surface CD4-independent stages of virus binding to the macrophages. Alpha-fetoprotein was found to inhibit the binding of HIV specifically at the V3 loop clade consensus peptide, and it interfered with viral postbinding events during HIV-1 infection of primary macrophages. Furthermore, a carbohydrate chain–related inhibition of HIV infection was found, which depended on the cell type (macrophage) and on differences in the glycan structure of the specific co-factor receptors involved in HIV entry into cells (213). Subsequently, it was shown that HAFP specifically interacts at the primary macrophage cell surface and competes with the gp 120 V3C binding of HIV-1 to these cells (214). Antibodies to the CCR-5 chemokine receptor inhibited AFP binding to these macrophages. Native AFP (not heat denatured) specifically interacted with electroblotted V3C-bound ligand, the CCR5 cell surface receptor. Thus, these authors' data indicated that the AFP inhibitory effect during HIV infection was related to an AFP-virus interaction, concurrent with HIV infection, that was related to AFP binding to the CCR5 family of macrophage chemokine receptors. This observation provides one possible explanation for the lack of vertical transmission of HIV-1 infection observed in the first and second trimesters of pregnancy (205).

Further data from the same French group revealed that HAFP bound to CCR5 receptors at both high- and low-affinity binding sites (K_A = 5.15 and 100 nM, respectively) localized on monocyte-derived macrophages (215). The CCR5 chemokine receptor is known to cluster with the CD4 receptor and to serve as a coreceptor for HIV intake and transfection (216–218). Both protein-to-protein interaction and lectin carbohydrate involvement were established as parts of the binding process; these experiments utilized treatments such as heat denaturation and neuraminidase exposure of AFP. As discussed above, HAFP was found to displace binding of the clade B HIV-1 gp 120 VC3 loop to the CCR5 receptor on the macrophage; conversely, CCR5 ligands were also able to displace AFP from its binding to the macrophage cell surface (219). Finally, it was shown that HAFP could bind to the CCR5 receptor expressed on HeLa cells, although not on HeLa cells lacking the CCR5 receptor. These data presented strong evidence that AFP binds directly to the CCR5 chemokine co-receptor associated with CD4 receptors expressed on primary macrophages (monocyte derived) and on transfected CCR5 HeLa cells.

AFP and Proteomics

The field of proteomics is defined as the study of the protein products of the genome and of their interactions and functions. In a similar fashion, the proteins expressed at a given time in a given environment constitute a proteome (220). The protein under study, AFP, is just one protein out of the entire proteome of cells that are undergoing development. Proteomics relies on the determination of cellular function and regulation through large-scale (array) measurement of protein function and interaction (221, 222). Fortunately, studies on the detection, structure, function, and regulation of AFP have benefited from experiences of a

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Hybritech enzyme-immunoassay in panel C. Note that human albumin (ALB; panel B) also bound to FA in a similar dissociation pattern, demonstrating a similar low affinity for FA as described in Ref. 151. The RIA for measurement of FA was obtained from Diagnostic Products Corporation as a solid-phase, no-boil assay.

30-year head start on the emerging field of proteomics. Although the *in vivo* functions of AFP still remain imprecisely understood, studies in the 1990s shed considerable light on the multiple physiological roles that AFP can play (11, 16, 18). In the realm of molecular structure research, cloning of the AFP gene and subsequent amino acid sequencing constituted focal points from which a wealth of information could then be probed from the gene bank. Concomitantly, investigations were reported in which the isoforms, epitopes, and conformational variants of AFP were being enumerated (17). Finally, regulation of the AFP gene has long served as an investigational tool of researchers who are seeking to uncover the mechanisms of gene suppression or quenching (silencing) of protein expression in the juvenile-to-adult transition.

The study of proteomics presents researchers with a formidable challenge for a number of reasons. First, protein levels vary widely, according to both cell type and environment. For example, AFP in the fetal compartments is present in mg/ml concentrations, while maternal SAFP concentrations differ by several log orders of magnitude (i.e., ng/ml). Second, unlike genomics, a field in which the researcher can amplify genes using the polymerase chain reaction (PCR), protein science has no comparable amplification method by which to aid the study of low-abundance proteins (223). In this respect, AFP has an advantage, namely, that it is naturally produced in high concentrations by the developing embryo/fetus. In addition, it can be synthesized in abundant quantities in recombinant systems such as *Escherichia coli,* yeast, the insect bacculovirus system, and transgenic rodents and livestock (i.e., goats). Third, proteomics is limited by the fact that the absolute quantity of protein that is synthesized is not a key feature because protein activities are tightly regulated post-translationally. Therefore, a protein can be abundant yet possess minimal physiological activity (e.g., ALB). In contrast, HAFP synthesized in transgenic mouse models of systemic arthritis (224) and autoimmune myasthenia gravis (225) has been shown to have an ameliorative effect on these diseases, as does native AFP during human pregnancy. Finally, because proteins interact functionally *in vivo,* protein-protein and protein–small molecule interactions need to be evaluated in processes of biomedical interest to researchers. Alpha-fetoprotein has already been demonstrated to act synergistically with cytokines and peptidic hormones and to interact with small molecules, such as estrogens, fatty acids, heavy metals, and drugs (11, 16).

From a technological standpoint, traditional proteomics involves separation of the proteins in a proteome, coupled to a means of identification. Until recently, the tools of choice were two-dimensional gel electrophoresis (2DGE) for separation and mass spectroscopy for protein identification (226, 227). Both of these methods have been extensively used for the identification of AFP in saline tissue extracts, cell lysates, tissue brei, and biological fluids (i.e., sera, AF, urine). Alpha-fetoprotein extracted from cell culture media has been also been well utilized (228, 229). However, 2DGE often fails, for one of several reasons. First, 2DGE does not work well for the separation of membrane proteins, which represent nearly 50% of the important molecular targets (230). Although the separation of membrane-bound AFP has been unsuccessful, the isolation of the cell-surface receptor for AFP has been achieved by isolation of cell membranes from human breast adenocarcinomas (95). Second, proteins of low abundance may be underrepresented in a 2DGE analysis, yet they often represent key players in sites of biological regulation. As ontogeny proceeds, the gene for AFP is downregulated and partially masked, resulting in a serum concentration of AFP that is barely detectable at 5 ng/ml (231–233). Thus, the function and structure of the true AFP form in the adult will be extremely difficult to elucidate. Although 2DGE is powerful, researchers wishing to apply proteomics to the discovery of molecular targets must seek ways in which to measure both protein abundance and biological activity. Alpha-fetoprotein is one among a select group of proteins for which an activity-based growth bioassay (nonenzymatic) has been developed (165, 171, 234, 235). Therefore, emerging technologies employing proteins/peptides will require high-throughput automation and techniques linked to protein array methods, isotope encoding, two-hybrid systems, cyberspace information technology, and activity-based methods in order to advance the utility of proteomics in biomedical research. In this fashion, the evolving proteomics of AFP will be gradually unveiled over the coming years. Due to the existence already of many years of study on its structure and function, AFP is a protein well suited to cross over the threshold into the exciting field of proteomics.

Concluding Remarks

The fetal form of AFP was first detected in 1957 (1) as a fetal-associated protein and later in 1963 (4) as a tumor-associated protein. During the 1970s, the isolation, purification, and characterization of AFP from a multitude of mammalian species were reported, leading to the development of immunoassays for both research and clinical use. With the advent of monoclonal antibodies in the 1980s, a widespread use of monoclonal-based assay platforms was made in commercial diagnostic kits. As a result of the discovery of the growth regulatory properties of AFP, it became apparent that this fetal protein represents an untapped resource for the armamentarium of biological therapeutics. Once an AFP linkage to fetal growth had been demonstrated, it was not long until studies of the growth modulatory properties of AFP were undertaken in multiple mammalian systems.

Studies of the biology of AFP blossomed in the mid- to-latter 1980s, especially in reproductive studies involving pregnancy and perinatal development. Concurrent with elucidation of the biological role of AFP involving fetal

growth in the 1990s, the therapeutic use of AFP itself loomed on the investigational forefront. By the end of the 1990s, the concept of AFP as a modular cassette protein had been proposed (16) and implemented by peptide fragmentation studies. Not only were individual AFP domains shown to possess biological activities, but the diverse biological properties of small amino acid fragments also were elucidated. The entire AFP molecule is now known to house motifs that display properties that may differ from the "parent" molecule (17). Classic examples of such motifs are the AFP-growth regulatory peptides first reported by Mizejewski (237), which are segments derived from the larger-molecular-weight "parent" AFP molecule. Such peptidic segments may participate in a host of biologic roles, including substrate antagonism and agonism, hemostasis, feedback control, and hormonal regulation. The future of AFP biotherapy may well lie in the judicious use of such AFP-derived peptides for drug targeting, diagnostics, and therapeutics.

An immunoassay kit developed for AFP peptides has already shown promise as a marker for fetal growth retardation (238). However, until well-characterized monoclonal antibodies are utilized to study the various AFP isoforms present in postnatal and adult life, it will remain uncertain whether the 70-kDa form that we presently measure is indeed the major adult form of AFP. Surprisingly, the major adult form of AFP in the rat is not the 70-kDa molecule (16). It is becoming increasingly evident that high AFP concentrations in adult males and nonpregnant females are coincident with abnormal growth and disease states (i.e., cancer, hepatitis, cirrhosis). Thus, one could propose that all present commercial kits for HAFP measure only the fetal and/or pathological tumor form rather than the adult form (whatever that may be).

Since HAFP is a largely a growth-promoting substance, the therapeutic injection of full-length (70 kDa), native AFP into normal and/or diseased adult could potentially be hazardous and would require extensive preclinical toxicity testing. The entire full-length, native AFP molecule is bristling with innumerable potential biologically active sites (eptotopes), some of which may not be fully exposed. Worldwide workshops on HAFP monoclonal antibodies have confirmed that such epitopes exist (239), and these molecular sites could be exposed in differing biochemical/biophysical microenvironments. On stimulation, the biologic response of these individual sites cannot be predicted or controlled and could produce undesired and/or dangerous side effects. This was precisely the reason why the United States Food and Drug Administration banned the sale and distribution of the human AFP immunoassay kits (reagents) as a medical device in 1971, a ban that endured until 1984 (17). It is with 40 years of experience of research in the field of AFP that this warning can still be issued. Unless under developmental stage control, full-length native AFP (70 kDa) can be viewed as a biological "loose cannon." Therefore, site-specific AFP-derived peptides offer a safer, more conservative approach for possible modulation of these multiple biological responses.

Notwithstanding the precautions stated above, future scientists in this field will be "mining" the entire AFP molecule for potential peptidic therapeutic drugs and mimics for use in a variety of diseases, such as myasthenia gravis, systemic lupus, thyroiditis, multiple sclerosis, arthritis, and other inflammatory/autoimmune diseases. The AFP peptides (see above) may well be utilized to seek out and identity molecular targets for therapeutics interventions of such diseases. Finally, the concept of AFP as a target for gene therapy and cancer vaccines, especially for AFP-producing hepatomas, currently lies at the threshold of biomedical utility (18).

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