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REVIEW

Structural and Functional Mapping of α-Fetoprotein

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Abstract—α-Fetoprotein (AFP) is a major mammalian oncofetal protein, which is also present in small quantities in adults. It is a member of the albuminoid gene superfamily, which consists of AFP, serum albumin, vitamin D binding protein, and α-albumin (afamin). Although physicochemical and immunological properties of AFP have been well-studied, its biological role in embryo- and carcinogenesis and in adult organisms as well as mechanisms underlying its functioning remain unclear. During the recent decades, the biological role of AFP has been evaluated by identification of its functionally important sites. Comparison of primary structure of AFP and some physiologically active proteins revealed similarity of some polypeptide regions. This has been used for prediction of AFP functions (i.e., its multifunctionality). Localization of functionally important sites followed by determination of their amino acid composition and type of biological activity has provided valuable information for structural–functional mapping of AFP. Some peptide fragments of AFP have been synthesized and tested for biological activity. This review summarizes data on structural–functional interrelationships. We also describe functionally important AFP sites found by various groups during the last decade of structural–functional mapping of AFP with experimentally confirmed and putative biologically active sites.

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Key words: AFP, α-fetoprotein, peptide fragments, structural–functional mapping, multifunctionality

α-Fetoprotein (AFP) is a major serum protein typical for the embryonic period of the development of all mammals (and possibly all vertebrates). AFP is also the best known tumor marker highly specific for primary liver cancer and teratoma [1-4]. Being discovered almost half a century ago, it is still an attractive object for intensive studies. Human and some animal AFPs (including those of mouse, rat, rabbit, guinea pig, etc.) have been isolated, purified, and characterized (see for review [5-7]). The primary structure of AFP has been reported for nine mammalian species (Swiss-Prot, TrEMBL databases). However, the biological role of AFP during embryonal development and in the adult organism (under normal conditions and during carcinogenesis) still remains unclear.

Modern databases (SwissProt, TrEMBL, PIR, etc.) and computer programs (BLAST, FASTA, ClustalW, etc.) provide the necessary tools for comparison of primary structures of proteins and peptides required for identification of similar sequences and functionally important sites. It is suggested that proteins exhibiting similar (identical) functions are characterized by the presence in their primary structures of sites with similar amino acid sequences underlying these functions. Usually these are short segments of the polypeptide chain containing up to 10-15 residues. Using analysis of structural–functional interrelationships, it is possible to predict putative protein functions by searching for amino acid sequences similar to those in known physiologically active proteins.

At the beginning of the 1990s, Mizejewski proposed the hypothesis of the "modular cassette" [8, 9]. According to this hypothesis, the AFP molecule contains a set of structurally and functionally different elements in short amino acid sequences tightly packed in the polypeptide chain. Signal sites similar to heterodimerization motifs of nuclear receptors for steroid and thyroid hormones were proposed as such structural elements recognized in human AFP. Based on data of high affinity of mouse and rat AFPs to estrogens [10-13], primary structures of AFP and estrogen receptor α (ERα) have been compared. This revealed that the C-end of a DNA binding domain of ERα contains a binding site for heat shock proteins (HSP), which is located near the estradiol-binding site. Human AFP contains a peptide segment of 34 residues similar to functionally important sites of HSP-70 [14]. Later this segment was synthesized chemically using the solid phase chemistry and defined as P149. Biological testing revealed that it inhibits estrogen-dependent cell

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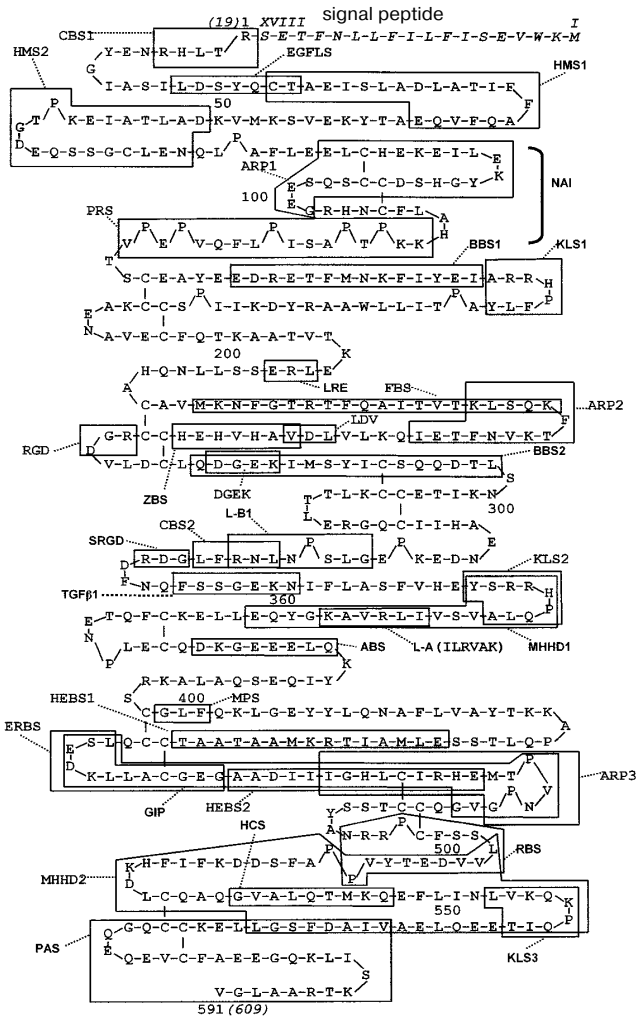

Structural–functional map of human AFP (the primary structure of AFP and positions of active sites are given in accordance with [7] with our additions and corrections; numeration of amino acid residues in the mature AFP molecule is given in brackets). Designations: I-XVIII) the signal peptide; EGFLS) epidermal growth factor like segment (residues 14-20); TGFβ1) segment of transforming growth factor β1 (residues 324-330); GIP) growth inhibitory peptide (residues 446-479); FBS) fatty acid-binding site (residues 209-228); HEBS1) primary human estrogen-binding site (residues 428-449); HEBS2) secondary human estrogen-binding site (residues 458-471); ERBS) estrogen-receptor-binding site (residues 446-457); CBS1 and CBS2) cyclin-binding segments 1 and 2 (residues 1-5 and 312-316); BBS1 and BBS2) bilirubin-binding sites 1 and 2 (residues 135-148 and 261-277); ABS) actin-binding site (residues 377-384); HMS1 and HMS2) heavy metal-binding sites 1 and 2 (residues 19-39 and 51-71); NAI) non albumin identity site (residues 30-120); PRS) proline rich segment (residues 111-129); KLS1, KLS2, and KLS3) kinetensin-like segments 1, 2, and 3 (residues 148-156, 340-348, and 538-546); LRE, LDV, RGD, DGEK, and ILRVAK) amino acid sequences (extracellular matrix protein motifs) (residues 195-197, 242-244, 253-255, 262-265, and 352-356); SRGD) segment reverse to RGD amino acid sequence (residues 318-320); MHHD1 and MHHD2) motifs of hetero- and homodimerization (residues 340-361 and 497-560); HCS) histocompatibility class II segment (residues 524-532); MPS) milk peptide segment (residues 399-401); PAS) plasminogen activator segment (residues 553-591); ZBS) zinc(II)-binding site (residues 244-250); RBS) human AFP receptor-binding site (residues 489-504); L-B1) laminin B1 segment (residues 307-314); L-A) laminin A segment (residues 352-357); ARP1, ARP2, and ARP3) apoptosis related polypeptides 1, 2, and 3 (residues 79-102, 224-237, and 463-478)

proliferation and growth of estrogen-dependent tumors. So it has been termed growth inhibitory peptide (GIP). Subsequent studies of human AFP primary structure revealed amino acid stretches similar to those of functionally important sites of some growth factors and protein hormones, inhibitors of cyclin-dependent kinases, extracellular matrix proteins, etc. [7-9, 14-19]. The presence of amino acid stretches in AFP that are similar to those in physiologically active proteins implies that it may have similar functions. Overall, AFP contains more than 20 types of functionally important sites. Some activities have only been predicted, and others have been experimentally confirmed. For mapping of biologically important sites of human AFP, some scientists use the numeration of the full-length, immature, molecule, whereas others use numeration of amino acid residues of the mature molecule lacking its signal peptide. Sometimes numeration of the mature molecule does not take into consideration the latest specifications of amino acid composition, which clearly indicate that the AFP molecule contains 591 rather than 590 residues (as it was believed earlier [20]).

In this review, we have tried to uniform the numeration of amino acid residues of peptide fragments of human AFP using numeration of the mature molecule and taking into consideration recent specifications (SwissProt 02771). We have summarized and analyzed data on structural–functional interrelationships and also described the most studied biologically active AFP sites revealed by different groups during the last decade. The figure shows the structural–functional map of human AFP.

STRUCTURE OF α-FETOPROTEIN

α-Fetoprotein was discovered in 1956 by Bergstrand and Czar using paper electrophoresis of serum proteins of human fetus; AFP was defined as a fraction exhibiting mobility of α-1-globulins [21]. In 1961 Muralt and Roulet identified AFP immunochemically as embryonic protein [22]. Interest in AFP significantly increased after its discovery in adults during carcinogenesis. In 1959, G. I. Abelev and his colleagues detected an antigen specific for chemically induced mouse hepatoma. In 1962, this antigen was discovered in amniotic fluid, liver tissue extracts, and blood serum of mouse embryos. It became clear that hepatoma cells produce and secrete embryo-specific α-globulin into blood. In the same year, these data were discussed at the VIII World Congress on Cancer

and published one year later [1]. In 1963 Yu. S. Tatarinov reported about discovery of embryo-specific α-globulin (defined later as α-fetoprotein, AFP) in blood of patients with primary liver cancer [2].

During embryonic development, AFP is mainly synthesized by fetal liver and visceral endoderm of the yolk sac. It may also be synthesized by embryonic kidney, pancreas, and gastrointestinal endoderm [23-25]. AFP concentrations increased and reached maximal values (10 mg/ml, ~10^{-4} M) in human fetal blood at 12-16 week of gestation. After this period AFP level sharply decreased and at birth it did not exceed 0.1 mg/ml. Under normal conditions, AFP concentration in serum of adult humans is about 5-10 ng/ml (~10^{-10} M) [26-28]. Changes of AFP level in maternal serum provide a screening test for some fetal disorders. For example, significant increase in AFP level is found in fetal neural-tube defects and its reduction is typical for Down syndrome [27, 29, 30]. Increase in AFP level in blood serum of adults indicates the development of various pathological processes, first of all malignant tumors, such as primary liver cancer and teratoma [3, 4, 27]. Increased AFP level was also detected in 15% of patients with acute hepatitis, in some cases of stomach cancer and pancreatic blastoma, and also in patients with chronic hepatitis and liver cirrhosis [31-35].

AFP is a glycoprotein containing up to 3-5% carbohydrates. Its molecular mass varies from 68 to 73 kD depending on carbohydrate content and species of animals used for isolation of this protein. The amino acid sequence of human AFP was deduced from nucleotide sequence of its mRNA [36] and the translation product of this mRNA contained 609 residues (SwissProt P02771). Initially it was demonstrated that during processing the newly synthesized polypeptide chain of AFP undergoes cleavage of signal peptide of 19 residues, and this yields the mature AFP molecule (of 590 residues). However, later it was demonstrated that in the AFP molecule isolated from cultural medium of human hepatoma HepG2 cells producing this protein the N-terminal amino acid is arginine, which was not included into the mature molecule before [20]. Thus, processing of immature AFP is accompanied by cleavage of an 18-residue peptide and therefore the mature human AFP molecule contains 591 residues. Subsequently, these data were also confirmed for embryonic AFP [37]. Molecular masses of members of the albumin protein family (which includes AFP) vary from 66 kD (albumin) to 82 kD (afamin). The differences in molecular masses are mainly attributed to the carbohydrate component. AFP contains approximately 4% carbohydrates and serum albumin contains just 0.5% carbohydrates, whereas vitamin D-binding protein and afamin have 5 and 21% carbohydrates, respectively [7, 38]. These proteins exhibit considerable similarity of their primary structures with characteristic distribution of cysteine residues; for example, AFP and serum albumin share 39% identity [38-40]. Proteins of this family also have similar α-helical secondary structure (up to 65-67% α-helices in the AFP molecule and about 50% in the albumin molecule) and they lack β-structure. These proteins also have similar spatial organization and consist of three homologous domains (I-III) each of which consists of two globular subdomains (IA, IB, IIA, IIB, IIIA, IIIB) linked by 15 regularly distributed disulfide bonds [41-43]. The three domain structure of the AFP molecule was demonstrated by electron microscopy, which revealed the existence of a U-shaped structure with three regions of electron dense contour masses: one on the top and two others on the periphery of the molecule [41]. Domains I, II, and III have similar secondary structure and contain 68, 55, and 71% α-helices, respectively; but they differ by parameters of tertiary structure [37]. Domains I and III have rigid tightly packed tertiary structure, and joined together by flexible domain II; its conformation corresponds to the shape of a molten globule form (MGF). The C-terminal part of domain II could be considered as a "hinge region", responsible for conformation flexibility of all domains, and therefore it promotes the interaction of AFP with ligands and other proteins [37, 41]. This site is characterized by lack of disulfide bonds and has been found only in the AFP molecule but not in other proteins of this family. It was shown that a fatty acid binding site of human AFP molecule is localized in domain II (its conformation corresponds to MGF); this site is located near Lys224 and the glycosylation site Asn233 [36, 44]. However, due to difficulties in crystallization, analysis of spatial structure by means of X-ray analysis and NMR spectroscopy is a rather difficult task. Mechanisms underlying AFP folding as well as forces inducing folding and unfolding of the protein globule remain unknown. The use of intensively developing methods of computer modeling might help to solve this problem.

Human AFP domains I and II include 186 residues each (residues 2-187 and 194-379, respectively); domain III consists of 192 residues (386-577). The highest identity of primary structures of human AFP and albumin is found in domain II (48%), whereas the lowest identity is found in subdomain IA (16%) [6, 38]. Comparison of primary structures revealed that polypeptide chains of human, rat, and mouse AFPs share high similarity. For example, primary structures of human and mouse AFP share 66% identity [36]. Primary structures of corresponding domains (e.g., domain I of human and mouse AFPs) also demonstrate high similarity. For AFP from various animal species the highest identity was characteristic for domain III (72%) and the lowest for domain I (59%). Interestingly, some domains of AFP and albumin from the same animal species exhibit lower similarity (to 28% identity) than corresponding domains of different animal species [45, 46].

Pairwise comparison of primary structure of AFP and albumin from various animal species revealed that

identity degree between these proteins reduced from man to dog, horse, mouse, and rat [47]. For human AFP and albumin the number of identical amino acid residues is 236 (38.8%), for dog AFP and albumin this value is a bit lower, 228 (37.5%), and then it decreases in the following order: horse (205, 33.7%), mouse (203, 33.5%), and rat (193, 31.7%). Consequently, a divergence vector of AFP and albumin from a common ancestor increases in this order and demonstrates the highest conservation for the pair of human AFP–albumin.

AFPs isolated from various tissues of one animal species (both embryonic and tumor) are characterized by the same amino acid sequence; they are also immunologically identical. However, numerous studies revealed that AFP exhibits molecular microheterogeneity, which is determined by differences in carbohydrate moiety of AFPs synthesized in different tissues. This determines the existence of AFP isoforms differing by p*I* values and lectin binding capacity [48-50]. Crossed affinity immunoelectrophoresis revealed the existence of up to ten glycoforms of human AFP [51]. The carbohydrate moiety may include glucose, galactose, mannose, N-acetylglucosamine, and sialic acids [38]. For example, branched oligosaccharide chain of human AFP contains two residues of sialic acids, D-galactose, and D-mannose. Certain lectins exhibit specificity not only to certain monosaccharide residues but also to the whole carbohydrate component. Human AFP contains only one glycosylation site, Asn233, whereas mouse AFP contains three such sites: the carbohydrate components are attached to Asn232, Asn310, and Thr483. Rat AFP also has three glycosylation sites: Asn232, Ser96, and Asn310 [52]. Tissue and tumor specificity of various glycoforms of AFP is determined by a tissue specific set of enzymes involved in glycosylation reactions. Content of various AFP glycoforms in embryonic and tumor tissues may be used for differential diagnostics of tumors and defects of fetal development [48].

POSSIBLE FUNCTIONS OF α-FETOPROTEIN

In spite of intensive study, many functions of AFP are still rather mysterious and contradictive. In various experimental models *in vivo* and *in vitro* human AFP and AFP from some animal species exhibit various biological activities [10-12, 53-78]. However, data demonstrating different biological activities of AFP are incomplete and rather contradictory. In our viewpoint differences in functional activity of AFP preparations obtained by various groups may be attributed to different isolation and purification procedures used and also to various degree of AFP (micro)denaturation and presence of various contaminations in them. Below we analyze briefly the main types of physiological activities of AFP, which suggest its putative functions.

Binding of hydrophobic ligands. AFP may bind various hydrophobic ligands. Embryonic and tumor cells, as well as human T-lymphocytes can accumulate unsaturated fatty acids bound to AFP [53-58]. So it was suggested that one of AFP possible functions may be transport of fatty acids into actively proliferating cells, which require increased energy supply and intermediates of fatty acid metabolism. In addition to fatty acids (mainly polyunsaturated acids, such as arachidonic, $C_{20:4}$, and docosahexaenoic, $C_{22:6}$), AFP from various sources may bind and transport metal ions, estrogens, bilirubin, retinoids, flavonoids, phytoestrogens, exotoxins, various dyes, and also drugs [56-69].

Estrogen binding activity of AFP attracts special interest because this may represent an important regulatory mechanism. Binding estrogens *in vivo*, AFP may be involved in regulation of concentration of free, active form of hormones. (This might protect fetal tissues against circulating maternal hormones.) Transporting estrogens to organs and tissues AFP may also prevent their degradation. Highly effective binding of both free and immobilized estrogens was initially demonstrated for mouse and rat AFP only [10-13]. Using affinity chromatography, we demonstrated that human AFP isolated from biological material by mild butanol extraction was able to bind (with high efficiency and affinity) immobilized estrone and the synthetic estrogen diethylstilbestrol (DES) [66-68]. Preincubation of butanol extract of the biological material with free estrogens and other steroids did not reduce AFP binding to immobilized hormones. However, preincubation with free DES was accompanied by almost 2-fold reduction of that interaction. This suggests that AFP binds to both free and immobilized hormones. There was strong binding of AFP to the immobilized hormone, because AFP was eluted from the column only by 10% butanol in 0.01 M veronal-medinal buffer, pH 8.6 (and was not eluted by 1 and 2 M NaCl and various organic solvents). Length of spacer and a mode of immobilization (i.e., spatial orientation of the hormone molecule) were crucial for such binding. It is possible that the interaction of AFP with estrogens *in vivo* requires some protein messenger. It is also possible that butanol causes dissociation of AFP complexes with estrogens and/or fatty acids and this releases ("clears") estrogen-binding sites. We suggest that removal of fatty acids results in conformational changes of the AFP molecule followed by formation of MGF and partial unfolding of the protein globule. Such conformational rearrangements would result in increasing of susceptibility of hydrophobic estrogen-binding sites for interaction with these hormones. Conformational changes may also occur during AFP interaction with an immobilized hormone or they may be due to AFP interaction with functional groups of the sorbent. However, molecular mechanisms underlying AFP binding to estrogens and other hydrophobic ligands still require further investigation.

Immunosuppressive activity. Although some reports indicate lack of immunoregulating activity of AFP [70], results of numerous studies show immunosuppressive activity of AFP. Good experimental evidence exists that AFP effectively interacts with macrophages, and this is accompanied by subsequent decrease in phagocytic activity of these cells and expression of Ia antigen; it also inhibits activity of natural killers (NK), suppresses proliferation of T-lymphocytes stimulated by concanavalin A (ConA) and phytohemagglutinin (PGA), and induces their suppressor activity [71-78]. Some authors believe that immunosuppressive activity of AFP is not an intrinsic property of the protein molecule, and it depends on the presence of low molecular weight ligands or carbohydrate components bound to the protein molecule. For example, it was found that only one of seven molecular variants of AFP differing in content of sialic acids and p*I* values exhibited immunosuppressive activity [79]. This isoform (of p*I* 5.1) representing 6% of total AFP level contained 1 mol/mol sialic acids. Others demonstrated that immunosuppressive activity of AFP (including a recombinant protein) is an intrinsic property of this protein [80].

We have confirmed immunosuppressive activity of AFP in cultured lymphocytes [81-83]. Using indirect immunofluorescence and commercially available monoclonal antibodies (MAB), we demonstrated the effect of human AFP on expression of surface activation antigen in lymphocytes from patients with bronchial asthma [82]. Human AFP caused 2-fold reduction in number of IgM-producing lymphocytes and nearly 2-fold increase in number of lymphocytes containing CD16 antigen. It also reduced expression of early and late activation antigens (CD25, CD71, HLA-DR) and some increase in expression of early activation marker CD95. Synthetic peptide fragment of human AFP LDSYQCT (residues 14-20) significantly decreased expression of HLA-DR antigen and induced expression of CD95. In blast transformation reactions, the heptapeptide (10^{-7}-10^{-9} M) caused moderate stimulation of proliferation of intact lymphocytes and marked inhibition of proliferation of PGA-activated lymphocytes [83]. Thus, the fragment of human AFP as well as the intact protein molecule exhibits immunosuppressive properties, and so this fragment can be considered as its biologically active site. These data suggest that immunomodulating properties of AFP are determined by the structure of its protein part and are realized irrespectively to the presence of ligands and/or carbohydrate components.

It is suggested that the immunosuppressive effect of AFP is mediated by receptor on the surface of immunocompetent cells. Different immunoreactive cells can recognize and specifically bind AFP [84-87]. Rat peripheral macrophages and monocytes of human peripheral blood and mouse activated lymphocytes have surface AFP binding receptors of 62-65 kD. In general, two types of receptors have been found on the surface of immunocompetent cells: one type is characterized by high specificity and low binding capacity, whereas the other has low affinity and high binding capacity. However, AFP receptors are not well characterized and their primary and spatial structures and also structure of their coding genes remain unknown.

Regulation of cell proliferation and tumor growth. Synthesis of AFP during embryonic development and its high concentrations detected in fetal serum suggest that this protein may stimulate tissue growth. Several studies have demonstrated that AFP can stimulate proliferation and differentiation of various cell types including lymphoid and epidermal cells, fibroblasts, hepatocytes, and ovarian and uterine cells [88-91]. However, data on the regulation of proliferation and differentiation of cells by AFP are very contradictory. Data accumulated during the last decade argue that AFP can regulate tissue growth and exhibit both stimulatory and inhibitory effects. For example, AFP inhibited *in vitro* and *in vivo* estrogen-dependent proliferation of immature mouse uterine cells, growth of estrogen-dependent MCF-7 human breast cancer cells, and human prostate cells [92-94]. Inhibition of tumor growth was also confirmed by epidemiological studies demonstrating that pregnancy significantly reduced risk of mammary cancer in women; this effect persists for several years after delivery [95, 96]. Recombinant human AFP also inhibits growth of estrogen-dependent MCF-7 tumor cells [97, 98]. However, in estrogen-independent MDA-MB-231 tumor cells AFP did not exhibit antitumor activity.

The detailed mechanism of inhibition of estrogen-dependent proliferation of cells and tumor growth by AFP remains unknown. It has been suggested that this effect is mediated by AFP receptors located on the surface of normal and tumor cells, and AFP binding to this receptor is required for penetration inside cells. There are many reports on the existence of receptors on the surface of MCF-7 human breast cancer cells, human lung, stomach, ovarian, and prostate cancer cells, mouse T-lymphoma YAC-1, rat Morris 777 hepatoma and rabdosarcoma, and also on the surface of normal cells (lung and heart endothelial cells, cells of reproductive and immune systems) [84-86, 99-103]. Despite these studies, AFP receptors are still poorly characterized.

It is also suggested that regulation of cell proliferation and growth of tissues by AFP involves its effect on programmed cell death. There are data indicating that AFP can inhibit or induce apoptosis. In the mid 1990s, it was reported that in a culture of HL-60 cells, AFP and MAB 167H.1 against AFP receptor blocked programmed cell death [104]. MABs were selected by inhibition of AFP binding to tumor strain lines Ichikawa and TA3/Ha. Inhibition of AFP receptor expression was accompanied by induction of apoptosis, which was evaluated by morphological changes and cell shrinking, by DNA fragmentation, and by kinetic parameters. Subsequent studies

revealed that AFP-positive forms of stomach cancer are characterized by significantly lower apoptotic index than AFP-negative forms [34]. This seems to support data on apoptosis inhibition by AFP. However, in 1997, it was demonstrated that AFP may be responsible for resistance of hepatoma HepG2 cells to cytotoxic effect of tumor necrosis factor [105]. Subsequently it was shown that in concentrations exceeding (0.1-0.2 mg/ml) AFP induced apoptosis in various human tumor cell cultures [106, 107]. Induction of apoptosis involved a Ca^{2+} and protein kinase independent mechanism, which also did not require synthesis of RNA and protein. Addition of AFP to a culture of tumor cells caused release of cytochrome *c* from mitochondria into the cytoplasm, apoptosome complex formation, and activation of caspases 3 and 9.

Recently, using mutant mice lacking the *afp* gene, a Belgian group published results that question the role AFP in regulation of tissue growth during embryonic development [108]. Intercross matings between heterozygotes by mutant allele ($Afp^{+/-}$) gave rise to offspring of wild type ($Afp^{+/+}$), heterozygous ($Afp^{+/-}$), and homozygous ($Afp^{-/-}$) by this allele. All of the embryos were viable and apparently normal. Genotyping revealed AFP mRNA in liver extracts and amniotic fluid of wild type embryos and heterozygous mice. The *afp* transcripts and AFP were not detected in the liver and amniotic fluid of embryos of homozygous ($Afp^{-/-}$) mice. Thus, embryos of mutant mice developed normally in the absence of AFP. Intercross matings between wild type females and mutant homozygous males gave rise to healthy offspring, whereas intercross matings between $Afp^{-/-}$ females and $Afp^{+/+}$ males did not give offspring because $Afp^{-/-}$ females were sterile. This conclusion was also confirmed by results of anatomical studies demonstrating lack of *corpora lutea* in ovaries of $Afp^{-/-}$ females and also by results of endocrinological studies demonstrating lack of progesterone synthesis (i.e., disorder of the ovulation process) [108]. Nevertheless, the authors found the presence of Graaf follicles, normal level of estrogens, and also pituitary gonadotropic hormones (luteinizing (LH) and follicle-stimulating (FSH) hormones) in $Afp^{-/-}$ mice. So they suggested that lack of fertility in $Afp^{-/-}$ females may be attributed to lack of AFP responsible for transport of estrogens to brain tissue accompanied by deregulation at the level of the hypothalamic–pituitary axis by inappropriate steroid feedback regulation [108].

FUNCTIONALLY IMPORTANT SITES OF α-FETOPROTEIN

During the last decade, several groups have tried to investigate AFP functions by comparing its primary structure with primary structures of other proteins with known biological functions. Identification of short amino acid stretches in the primary structure of AFP similar to sequences of these proteins would suggest that AFP may share similar functions, and such stretches may represent biologically active sites of AFP responsible for some functions. This approach is well founded and widely used for prediction of functions of many proteins [109]. It is especially valuable when amino acid sequence of a protein of interest is known but functions are not understood at all. The applicability of this approach is also confirmed by the fact that serum albumin, a protein homolog of AFP, which shares high similarity with its primary structure, lacks sites exhibiting similarity with primary structures of physiologically active proteins being compared with AFP ([7] and our own unpublished results). This is quite reasonable because albumin does not demonstrate specific physiological functions. AFP and albumin share up to 50% identity only at bilirubin and unsaturated fatty acids binding sites; the latter confirms the similarity of transport functions of these proteins. The primary structure of AFP contains many short amino acid stretches (mainly up to 15 residues) with experimentally confirmed or putative biological functions (see figure). Demonstration of functional importance of some AFP sites in various tests *in vitro* and *in vivo* supports the applicability of this approach.

Heptapeptide LDSYQCT and cyclin-binding motifs. Taking into consideration data on a stimulatory effect of AFP on cell proliferation and tissue growth, we searched the primary structure of AFP for similarity with growth factors and some protein hormones. In 1997 comparing the primary structure of AFP and epidermal growth factor (EGF), several amino acid stretches exhibiting high similarity were found (table): the sequences LDSYQC and LDSYTC in AFP (residues 13-18) and human EGF (residues 26-31), respectively [110]. We suggest that the sequence LDSYQC of human AFP as LDSYTC in human EGF may represent a fragment of its receptor-binding site. EGF receptor (as well as insulin receptor) consists of an extracellular ligand binding α-subunit and intracellular β-subunit exhibiting tyrosine kinase activity [111]. Hormone binding to the α-subunit results in stimulation of the β-subunit tyrosine kinase activity triggering a cascade mechanism of activation of extracellular protein kinases. These kinases mediating hormone action are involved in regulation of numerous physiological functions by phosphorylation of various enzyme and transcription factors, as well as proteins regulating transmembrane transport of various substances, etc. Some sites of human AFP and insulin α-chain also share 33% identity: LDSYQCT (residues 13-19) and ENYCN (residues 17-21), respectively [15, 110]. After specification of human AFP primary structure (SwissProt 02771), the segment LDSYQCT was identified as residues 14-20 (i.e., heptapeptide LDSYQC includes residues 14-19). These peptides were synthesized chemically and tested in various biological tests. *In vitro* they stimulated in dose-dependent manner glucose uptake by erythrocytes isolated from

patients with diabetes mellitus [112]. Insulin pentapeptide (10^{-5} M) significantly increased glucose uptake by erythrocytes of healthy donors and erythrocytes of patients with diabetes mellitus type I (insulin-dependent) and II (insulin-independent), and at lower concentrations (10^{-7} and 10^{-9} M) the effect was less pronounced. AFP heptapeptide (10^{-5} M) significantly increased glucose utilization by erythrocytes from patients with insulin-dependent and insulin-independent diabetes. Heptapeptide LDSYQCT also exhibited immunomodulating properties. In blast transformation reaction, LDSYQCT (10^{-7}-10^{-9} M) caused moderate proliferation of non-activated lymphocytes and pronounced inhibition of proliferation of PGA-activated lymphocytes [113]. Maximal (2.2-fold) stimulation of spontaneous lymphocyte proliferation was observed at concentration 10 μg/ml. Inhibition of proliferation of PGA-activated lymphocytes (by 40%) did not depend on amount of peptide added. It is possible that *in vivo* this peptide suppresses lymphocyte proliferation increased in autoimmune diseases without any influence on normally proliferating cells [83]. In a culture of K-562 cells, the heptapeptide caused 1.5-2.0-fold increase in NK cytotoxic activity. Maximal effect was observed at peptide concentration 10 μg/ml. These data suggest that peptide LDSYQCT may increase antiviral and anti-infectional activity of lymphocytes. It was also demonstrated that this peptide exhibits dose–response inhibitory effect on proliferation of lymphocytes from patients with acute and chronic lymphocytic leukemia exhibiting low sensitivity to Cytosar; i.e., the heptapeptide increased antiproliferative activity of this preparation [114]. In cell culture of naturally activated lymphocytes from patients with infectious allergic myocarditis, this peptide significantly decreased expression of later activation antigen, HLA-DR, and induced expression of Fas-antigen (CD95). Increase in $CD95^+$ lymphocytes supports the hypothesis that heptapeptide LDSYQCT induces apoptosis because this process depends on Fas/FasL cell interaction [115]. Thus, one can conclude that the amino acid stretch LDSYQCT exhibits a significant proportion of the immunomodulating and other biological properties of the intact human AFP molecule and, consequently, may be considered as one of its biologically active sites.

Sites structurally similar to LDSYQCT have also been found in amino acid sequence of transforming growth factor β1 (TGFβ1), which also exhibits immunosuppressive activity and increases proliferation of some cells. Both proteins (AFP and TGFβ1) contain many cysteine residues (including double cysteines): they represent 8 and 5.4% of all amino acid residues of TGFβ1 and AFP, respectively. The sequences LDTNYC (residues 2-7) and LDTQYS (54-59) of TGFβ1 share 50 and 33% identity with AFP stretch LDSYQC, respectively [16, 116]. Two other sites found in these proteins differ by one amino acid residue; these are FSSGEKN (residues 324-330) in human AFP and FSSTEKN (residues 8-14) in TGFβ1. Biological properties of these peptides have not been studied yet. It is possible that peptide FSSGEKN represents an additional functionally active site of AFP (table).

In the human AFP molecule we found short amino acid stretches exhibiting similarity with structural motif RxL, typical for inhibitors of cyclin-dependent kinase (CKI, cyclin-dependent kinase inhibitor). These inhibitors belong to a family of Kip/Cip proteins [117].

Amino acid sequences of some growth factors exhibiting similarity with the fragments LDSYQCT and FSSGEKN of human AFP

Protein	Amino acid sequence	Identity, %	Conservative substitutions, %	Total similarity, %	Reference
AFP (residues 14-20)	L D S Y Q C T	7/7 (100)	7/7 (100)	100	[113]
α-Chain of insulin (residues 17-21)	E N Y – C N	2/6 (33)	3/6 (50)	83	[15, 110]
EGF (residues 26-31)	L D S Y T C	5/6 (83)	1/6 (17)	100	[110]
TGFβ1 (residues 2-7)	L D T N Y C	3/6 (50)	3/6 (50)	100	[16]
TGFβ1 (residues 54-59)	L D T Q Y S	2/6 (33)	4/6 (67)	100	[16]
Trophoblast β-globulin (various residues during various developmental stages)	Y E C E	2/4 (50)	2/4 (50)	100	[110]
	Y Q C E	2/4 (50)	2/4 (50)	100	[110]
AFP (residues 323-329)	F S S G E K N	7/7 (100)	7/7 (100)	100	[16]
TGFβ1 (residues 8-14)	F S S T E K N	6/7 (86)	0/7 (0)	86	[16]

Note: AFP, α-fetoprotein; EGF) epidermal growth factor; TGFβ1, transforming growth factor β1.

Activity of cyclin–CDK (cyclin-dependent kinase) complexes is known to determine progression of all phases of the cell cycle. Activity of CDK depends on many factors including phosphorylation/dephosphorylation processes, cyclin binding ability, and interaction with various protein CKIs, which impair phosphorylation of transcription factors and some other proteins required for cell division. It was shown that CKI caused accumulation of dephosphorylated retinoblastoma proteins (pRb) and E2F transcription factors followed by impairment of cell cycle and arrest of cell cycle at G_1 phase. Degradation of CKI involves ubiquitin-dependent pathway and proteasomes recognizing polyubiquitinated proteins as their substrates. Selective and strictly determined CKI degradation by proteasomes is a universal mechanism of cell cycle regulation in all eukaryotic cells; this mechanism provides "continuity and irreversibility" of mitosis, G_1 and S phases [117, 118]. Motif RxL is a cyclin-binding site of CKI; it is present in human and mouse p27 protein as RNL sequence (in both proteins these are residues 30-32). Identification of cyclin-binding motifs in AFP primary structure (figure) suggests the existence of structural preconditions required for involvement in regulation of cell cycle and cell proliferation. Interestingly, human AFP contains RxL motif in the RTLHR sequence (residues 1-5) and LNRFL (residues 312-316) as in right (RTL and RFL), and in inverted (LHR and LNR) state [47]. This RxL motif we also found in ubiquitin SwissProt P62988, where it exists as RTL (residues 54-56).

Growth inhibitory peptide (P149), its analogs, and the active fragment. In the mid 1990s, an American group identified a structural motif of human AFP responsible for suppression of estrogen-dependent proliferation of uterine cells of immature mice and estrogen-dependent tumors; they also demonstrated that this structural motif represents a stretch of 34 residues of domain III (see figure) [119-123]. In contrast to the intact AFP molecule, biological effect of this peptide did not require preincubation with hormone. This peptide was defined as growth inhibitory peptide (GIP) and designated as P149 (or P447). It has been synthesized chemically and well characterized. Results of a series of convincing experiments by Mizejewski and Jacobson's groups presented in many publications attract much attention. They directly confirmed putative AFP functions predicted by comparison of amino acid sequences of AFP and physiologically active proteins (followed by biological tests of amino acid stretches recognized).

In some reports, amino acid residues of GIP are numbered as 447-480. However, according to the latest numeration of residues in the polypeptide chain of mature human AFP the particular amino acid sequence of GIP corresponds to residues 446-479 (SwissProt 02771). Immunochemical studies revealed that antibodies to this peptide do not recognize the native molecule but they do interact with AFP molecules conformationally changed in the presence of high estradiol concentrations [119]. It is suggested that in the native AFP molecule, GIP is in hidden and sterically inaccessible state and it becomes susceptible during conformational changes of the protein molecule. Chemical modification of cysteine residues followed by formation of S-methylcysteine or S-(2-aminoethyl)-cysteine yielded peptides which were active in the test of inhibition of estrogen-dependent proliferation of mouse uterine cells. Activity of methyl- and aminoethyl-derivatives of P149 peptide was 31 and 29%, respectively, and it was comparable with activity of intact peptide (45%) [120]. Several other analogs of P149, in which two cysteine residues were replaced for alanine, glycine, or serine, have also been synthesized. The highest biological activity in the inhibition of estrogen-dependent proliferation test was exhibited by alanine-containing peptide, 37% [121-123]. The glycine-containing peptide was two-times less active (17%), whereas the serine-containing peptide lacks this biological activity. These peptides were inactive in the test of inhibition of prostate tumor growth [124].

Subsequent studies revealed that a P149 fragment, the octapeptide EMTPVNPG (residues 471-478), exhibits maximal biological activity. It caused more potent inhibition of mouse uterine cell proliferation (49%) than the whole P149 peptide (45%) and intact AFP (35%) [125]. Two synthetic analogs of this octapeptide have been synthesized [126]. In one of them two proline residues were replaced for two 4-hydroxyprolines, and the resultant octapeptide EMTOVNOG has higher hydrophilicity than the initial peptide. The second peptide, cyclo-EMTOVNOG, has higher hydrophobicity and more rigid conformation than the initial octapeptide. However, both peptides exhibited the same biological activity, which was similar to the intact octapeptide. It is suggested that P149 peptide exerts its biological effect via G-protein coupled membrane receptor. Data of molecular dynamics support this suggestion. It was shown that cyclo-EMTOVNOG can bind to surface GPR30 receptor; this receptor signaling involves G-protein [127]. Some growth factors contain fragments similar to P149, and effects of these growth factors are mediated by G-protein. Some fragments of GIP share similarity with amino acid stretches of human dopamine and gastrin receptors, rat somatostatin receptors, pig receptors for calcitonin, mouse receptors for insulin like growth factor II, fibroblast growth factor, etc. [123]. In an *in vivo* model, both P149 and the whole AFP molecule decreased fetotoxicity of estrogens and insulin [17]. (Defects in fetal development and fetal death during prenatal development decreased by 50 and 63-73%, respectively.) The sequence of GIP shares some similarity with some stretches of heat shock proteins and other stress proteins associated with osmotic and oxidative shock. These amino acid sequences include AXEEGXSQECIGKLCIQHE (residues 552-570) and AEEEGGSXE-

CIGELCLQHE (residues 533-551) of pea and petunia HSP-70 [17, 123]. Based on these data the authors suggest that GIP is a fragment of the AFP molecule sensitive to stressor treatments. It is possible that AFP is involved in protection of the fetus against various stress (shock) treatments and its sites corresponding to particular fragments of GIP are responsible for this function.

Sites for hydrophobic ligand binding. Several hydrophobic ligand-binding sites have been precisely mapped on the AFP molecule (see figure). Localization of an estrogen-binding site was determined using a chimeric protein constructed using human and rat AFP. It was shown that the main rat AFP estrogen binding site is localized in domain III and represents amino acid stretch of the sequence ELIDLTGKMVSIAST (residues 424-438) [63, 99]. A hydrophobic fragment in the middle of GIP, defined as P149b and representing the sequence AADIIIGHLCIRHE (residues 458-471) bound free 17β-estradiol [121]. It is possible that this peptide is the low affinity estrogen-binding site of human AFP (with AC_{50} of $1.6 \cdot 10^{-4}$ M). Lack of binding of free estrogens by native AFP may possibly be explained by the fact that this site is conformationally hidden and becomes susceptible only in the presence of estrogens. It has been shown that high estrogen concentrations (molar AFP to estradiol ratio of 1 : 1280) caused conformational changes of AFP [119]. Comparison of primary structures revealed that human AFP site ELMAITRKMAATAAT (residues 428-442) shares high similarity with the main estrogen-binding site of rat AFP [124]. The latter suggests that this segment may be the main high-affinity (K_d of about 10^{-8} M) estrogen-binding site of human AFP. The other human AFP site, which binds (ERα), is the initial fragment of P149 peptide of the sequence LSEDKLLACGEG (residues 446-457); being defined as P149a, it has affinity toward ERα of $9 \cdot 10^{-5}$ M [119].

The other functionally important site responsible for binding of unsaturated fatty acids has also been localized; it was shown that Lys224 is especially crucial for binding [44]. This site localized in domain II of human AFP includes the sequence MKNFGTRTFQAITVTKLSQK (residues 209-228). Based on comparison of primary structures of various fatty acid binding proteins (serum albumin, fatty acid synthase) and characteristic distribution of lysine (K) residues, the existence of similar sites in two other AFP domains was proposed [128]. These putative sites are: YKEVSKMVKDALTAIEKPTGD (residues 42-61) and KKAPQLTSSELMAITRKMAAT (residues 419-439) of domain I and III, respectively. They obviously meet the specified criteria.

Epitope sites. In Abelev's laboratory, it was found that one of the epitopes of AFP exists in a hidden form; it is recognized only after partial denaturation by adsorption on a nitrocellulose membrane (NCM) [129, 130]. Competitive immunoaffinity electrochromatography using a set of 51 MABs against human AFP epitopes revealed the following types of interaction of AFP–MAB complex with antibodies fixed on a NCM: 1) total neutralization; 2) partial neutralization; 3) unidirectional neutralization; 4) augmented binding; 5) lack of interaction [131]. These MABs recognized 23 various epitopes in the AFP molecule. These data were used for epitope mapping; it consists of at least eight individual epitopes and eight epitope clusters (A, B, C, D, E, F, G, and H), recognized by several MAB with common epitope specificity. Clusters A, B, and E were the largest by MAB susceptibility and they are referred to as immunodominant epitopes. Taking into consideration data on AFP fragmentation, it was suggested that the major part of the epitopes (clusters A, B, D) is located in domain III, whereas clusters E and C are localized in domain I and domain II, respectively [131]. Various conformational variants of AFP were recognized in dependence on susceptibility of clusters and individual epitopes; in the case of susceptibility, they are characterized by positive (+) reaction between AFP and corresponding MAB and lack of reaction (–) when a given epitope was insusceptible. Using this approach, the existence of two forms of human AFP was demonstrated. In one form, epitopes of cluster D were opened and susceptible (this is the epitope-positive form, D+), and in the other one these epitopes were hidden and inaccessible to MAB (epitope-negative form, D–). Similar properties were also found for epitopes 106, 108, and cde; and so these clusters were proposed as markers of conformational state of AFP. Epitope-positive and epitope negative forms of AFP have the same molecular mass and are interconvertible. For example, after addition of 0.2% sucrose 106+ and D+ were converted into 106– and D–. Amniotic fluid contains preferentially epitope-positive forms, whereas blood serum of patients with primary liver cancer and teratoma preferentially contain epitope-negative AFP forms [132].

Using immunoenzyme analysis and a set of 36 MAB, another group investigated epitope structure of human recombinant AFP containing one, two, or all three domains: domain I, domain II, domain III, domains I-II, domains II-III, or domains I-II-III [133]. There were 13 and 17 epitopes localized in domain I and domain III, respectively. One epitope recognized by MAB represented the AFY6 octapeptide CKAENAVE (residues 175-182). Interestingly, hidden epitopes induced spontaneous immune response in patients with primary liver cancer, liver cirrhosis, or chronic hepatitis. Patient immunoglobulins react with those epitopes, which are inaccessible in native AFP and become susceptible in the deglycosylated state. Some epitopes belong to antigen of major histocompatibility complex and can be recognized by $CD8^+$ T-lymphocytes. Stem cells obtained by gene engineering methods and producing AFP were able to generate AFP-specific immune response in a culture of autologous human lymphocytes and transgenic mice HLA-A2.1/Kb [134]. These T-lymphocytes recognized the nanopeptide

GVALQTMKQ of AFP (residues 524-532) (see figure). Lymphocytes activated by this nanopeptide recognized AFP-containing cells in cytotoxicity test and in the test of cytotoxin release [18].

Other putative functionally important sites. One of the mechanisms that may be responsible for putative AFP involvement in regulation of cell proliferation and tissue growth is modulation of transcription. Identification of homo- and heterodimerization motifs typical for nuclear receptors of steroid and thyroid hormones in all three domains of human and rat AFP (figure) seems to support this hypothesis; nuclear receptors of these hormones act as transcription factors [8, 9, 19]. These motifs promote protein dimerization, which is crucial for the spatial structure required for binding to DNA. Human AFP domain III contains motifs of thyroid receptor heterodimerization. These are the amino acid stretch YSSRHPQLAVSVILRVAKGYQE (residues 340-361) and a sequence of GIP (residues 446-479) [99, 119]. It is suggested that AFP may play the role of transcription cofactor by forming heterodimers with nuclear receptors and stabilizing their binding to hormone-responsive elements of DNA. Human AFP also contains sites that are similar to homodimerization motifs typical for steroid hormone receptors; these include residues 497-520 and 516-560 [9]. AFP might regulate transcription by inducing or repressing certain genes in dependence on its effect on transcription factor dimerization (where it promotes or blocks this process). This may result in either stimulation or inhibition of cell proliferation and tissue growth. Interestingly, human AFP sites similar to homo- and heterodimerization motifs are located in a segment that shares high homology with serum albumin. This may suggest that albumin has some structural preconditions required for involvement in regulation of cell proliferation and tissue growth. Comparative analysis of the primary structures of AFP and extracellular matrix proteins revealed that human AFP domain II contains short amino acid stretches (LRE, 195-197; LDV, 242-244; RGD, 253-255; DGEK, 262-265; ILRVAK, 352-356, etc.) that are similar to signal adhesive sites in laminin, collagen, and fibronectin [7, 52]. Identification of these motifs supports the hypothesis that AFP may regulate processes of proliferation, differentiation, and migration including inhibition of growth and metastases of tumors. Interestingly, the AFP sequence also contains many motifs of cell adhesion; this suggests the possibility that AFP may have some functions typical for extracellular matrix proteins.

All three domains of human AFP contain amino acid stretches similar to sites of homeodomain proteins controlling proper body development from the embryonic blastophylum [128]. Homeodomain proteins regulate formation of anterior–posterior direction and ventral–dorsal axis, body segmentation, and formation of neural tube and digestive channel. The amino acid sequence NEYGIASILDSYQCT (residues 6-20) of human AFP domain I is similar to the site of mouse Pou-domain protein, whereas stretches 224-238, 240-254, and 251-269 of domain II and 446-457, 472-481, and 512-553 of domain III share similarity with sites of homeodomain proteins of some animal species (see for review [128]).

Alignment of primary structures of AFP, cytokines, and proteins involved in regulation of apoptosis revealed that human AFP domains I, II, and III contain amino acid stretches exhibiting various degrees of similarity (up to 71% identity) with sites of tumor necrosis factor receptors (TNFRα), Fas and Bcl-2 proteins, and also of α-interferon and interleukins [135]. In other words, human AFP contains structural prerequisites required for involvement in regulation of apoptosis.

There is increasing evidence that a significant proportion of proteins exhibit multiple functions that are determined by their structure and interaction with ligands or with other proteins and factors. Such proteins possess several (or even numerous) functionally important sites determining their multifunctional properties [136, 137]. It is possible that proteins play the role of some reservoir or precursor of many biologically active peptides. Limited proteolysis of these proteins yields a pool of tissue specific peptides, which may be involved in regulation of activity of various systems. Polypeptide chains of these proteins may represent the mode of structural organization of such peptides, which provides their stability and longer lifetime.

Identification of many functionally active sites in the AFP molecule suggests that under certain conditions this protein may exhibit functions associated with these sites. We should emphasize that these are putative functions of AFP. These functions may be realized in the case of presentation of these sites on the surface of the AFP molecule. This requires conformational changes of the AFP molecule, which may occur during protein–protein, protein–ligand, or protein–membrane interaction. Decisive evidence demonstrating certain AFP function probably requires gene-engineering construction of AFP with altered functionally active sites and testing of the recombinant preparations in various biological models.

We suggest that the polypeptide chain of AFP is a natural reservoir supplying embryonic tissues with biologically active peptides such as LDSYQCT. In this case, functionally active AFP sites and biologically active peptides formed during intracellular proteolysis may be involved in regulation of various physiological processes including cell proliferation, tissue growth, immune system functioning, transport of hydrophobic ligands, etc. In adults, particularly in humans, concentrations of many growth factors and protein hormones (e.g., insulin) are as low as AFP (to 10 ng/ml, or ~10^{-10} M). So, this low AFP concentration may be functionally important.

A putative role of AFP in inhibition of carcinogenesis is supported by both experimental data and identifica-

tion of GIP in the primary structure of this protein as well as the amino acid stretches similar to those in cytokines and proteins involved in induction of apoptosis. AFP preparations are employed in chemotherapy of cancer [138]. Chemically synthesized peptides (representing fragments of AFP) that exhibit biomodulatory properties may also be used as a basis for development of peptide and non-peptide drugs.

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