Human α-Fetoprotein Primary Structure: A Mass Spectrometric Study†

Pietro Pucci,*,‡,§ Rosa Siciliano,‡,∥ Antonio Malorni,*,‡,∥ Gennaro Marino,‡,⊥ Mario F. Tecce,# Costante Ceccarini,# and Benedetto Terrana#

Servizio di Spettrometria di Massa, Consiglio Nazionale delle Ricerche, via Pansini 5, 80131 Napoli, Italy, Istituto Chimico, Università della Basilicata, Potenza, Italy, Istituto Chimica Molecole Interesse Biologico, Consiglio Nazionale delle Ricerche, Arco Felice, Napoli, Italy, Dipartimento di Chimica Organica e Biologica, Università di Napoli, Napoli, Italy, and Laboratorio di Biologia Cellulare, Centro Ricerche, Sclavo SpA, Siena, Italy

Received July 16, 1990; Revised Manuscript Received December 19, 1990

ABSTRACT: The amino acid sequence of human α-fetoprotein, a 67-kDa protein present in mammalian embryonic serum, was verified by fast atom bombardment mass spectrometric (FAB/MS) analyses of three different enzymatic digests of the protein. Human α-fetoprotein obtained from a large-scale cell culture was digested with trypsin and V-8 protease either separately on two different samples or combined on the same one. The V-8 protease digest of the protein was partially fractionated by HPLC; the other samples were directly analyzed by FAB/MS without previous purification steps. About 90% of the α-fetoprotein amino acid sequence was verified by mass spectrometric analysis; this also confirmed that the cell-derived protein is identical with the hepatoma-derived protein. FAB analysis revealed that the N terminus of the mature protein is arginine rather than threonine, with the threonine occupying the second position. Therefore, the processing site of the α-fetoprotein signal peptide during maturation of the protein occurs at the N-terminal side of the arginine residue formerly indicated as residue −1. Thus mature α-fetoprotein contains 591 amino acids rather than 590.

The major component of mammalian embryonic serum is α-fetoprotein. The synthesis of this protein decreases around the time of birth when albumin synthesis starts, and it becomes the major component of serum in postnatal life. In the fetus, α-fetoprotein is synthesized by the liver, yolk sac, and the intestine. In the adult, albumin synthesis is restricted to the liver; α-fetoprotein can be detected in sera of adult patients with hepatomas or teratomas and during pregnancy in cases of open neural tube defects. The genes for the two proteins are tandemly arranged, thus implying that the control of activation and shut off of the two genes must be differently coordinated in each tissue (Sell, 1982; Abelev, 1983; Urano et al., 1984).

The mRNA nucleotide sequences of mouse (Law & Dugaiczik, 1981), rat (Jagodzinski et al., 1981), and human (Morinaga et al., 1983) α-fetoproteins were determined, and the primary structure of α-fetoprotein was deduced from the cDNA sequence. Comparison of the amino acid sequences showed that human albumin and human α-fetoprotein share a high level of amino acid identity and a similar secondary structure can be predicted. The primary structure of α-fetoprotein, however, has never been directly determined, and confirmation of the cDNA sequence is thus lacking. Early attempts to determine the α-fetoprotein sequence yielded only limited N- and C-terminal portions of the protein (Aoyagi et al., 1977).

A method that allows the production and purification of α-fetoprotein from a human hepatoma cell line (Tecce & Terrana, 1988) has been developed by some of the authors; the scaling up of this method permitted the production of several grams of the protein. Since this protein may be well suited to serve as a standard for calibration in its clinical immunoassays, it was important to establish the homogeneity of the preparation. It has previously been shown that the product obtained by these methods is indeed α-fetoprotein and it is not contaminated with albumin (Tecce & Terrana, 1988) and that the product contains a single biantennary, fully sialylated oligosaccharide chain identical with that previously reported for tumor-derived α-fetoprotein (Terrana et al., 1990). However, the verification of the entire primary structure of this 67-kDa protein by classical methods would have needed a great commitment in time and large amounts of sample.

The development of mass-spectrometric-based procedures for the analysis of biopolymers, such as the FAB-mapping strategy (Biemann et al., 1982; Morris et al., 1983), provides the most important and complementary alternative, at present, to cope with this kind of protein structural problem. Since the use of fast atom bombardment mass spectrometry (FAB/MS)[1] in the verification and control of protein sequences deduced by cDNA sequences had already been reported for several proteins (Gibson & Bieman, 1984; Takao et al., 1984; Morris & Greer, 1988), the authors were encouraged to apply FAB mapping to the analysis of the primary structure of α-fetoprotein. This paper reports the verification of 90% of the entire α-fetoprotein sequence obtained by direct FAB/MS analysis of three different proteolytic digests of the protein, together with a partial HPLC separation of peptides followed by FAB/MS analysis of the fractions. Results revealed that the mature protein actually contains an extra amino acid at the amino terminus compared to the cDNA-derived sequence, thus suggesting that the site of processing of the leader peptide had been incorrectly placed in previous studies

† This work is supported by grants from the Consiglio Nazionale delle Ricerche, Roma, Italy (G.M. and A.M.), from the CNR target project Biotecnologia e Biostrumentazione, and from the Ministero della Pubblica Istruzione, Roma, Italy (G.M.). This paper is dedicated to Prof. Alessandro Ballio on the occasion of his 70th birthday.

* Address correspondence to this author.
‡ Servizio di Spettrometria di Massa, CNR.
§ Istituto Chimico, Università della Basilicata.
∥ Istituto Chimico Molecole Interesse Biologico, CNR.
⊥ Dipartimento di Chimica Organica e Biologica, Università di Napoli.
Laboratorio di Biologia Cellulare, Centro Ricerche, Sclavo.

[1] Abbreviations: FAB/MS, fast atom bombardment/mass spectrometry; DTT, dithiotreitol; TFA, trifluoroacetic acid; Cpase, carboxypeptidase.

0006-2960/91/0430-5061$02.50/0

of the cDNA (Morinaga et al., 1983; Gibbs et al., 1987).

Experimental Procedures

Materials. Human α-fetoprotein was purified from culture media of the Hep G2 cell line, as previously described (Tecce & Terrana, 1988). Protein purity was greater than 98%, as estimated by gel densitometry. Trypsin, V-8 protease, dithiothreitol (DTT), iodoacetic acid, glycerol, and thioglycerol were purchased from Sigma Chemical Co. HPLC-grade solvents and reagents were obtained from Carlo Erba. Sep-pak C-18 cartridges and the μ-Bondapack C-18 HPLC column (30 cm × 0.45 cm, 10 μm) were obtained from Waters.

Reduction and Carboxymethylation of α-Fetoprotein. Purified α-fetoprotein (1.5 mg) was dissolved in 300 μL of 0.3 M Tris-HCl, pH 8.0, containing 6 M guanidine HCl, 1 mM EDTA, and treated with DTT (10:1 molar excess with respect to the cysteine residues) at 37 °C for 2 h. The reduced protein was carboxymethylated by reaction with a 5:1 molar excess of iodoacetic acid solution (pH 8.0) to the putative total SH groups; the reaction was carried out at room temperature for 30 min in the dark. The protein was freed from salts and low molecular weight substances by passing the reaction mixture through a Sep-pak C-18 cartridge, after which it was lyophilized.

Proteolytic Digests. V-8 protease and tryptic hydrolyses were both carried out in 0.4% ammonium bicarbonate buffer at pH 8.0 and 8.5, respectively. When the two proteases were used together, the tryptic digest was performed first; the enzyme was then inactivated by boiling the reaction mixture for 5 min, and the sample was eventually submitted to V-8 protease hydrolysis. A total of 0.5 mg of protein was used for each experiment.

HPLC Separation of α-Fetoprotein Peptides. The portion of V-8 protease-digested α-fetoprotein was partially fractionated by HPLC with a μ-Bondapack C-18 column; solvent A was 0.1% trifluoroacetic acid (TFA) in water; solvent B was 0.07% TFA in acetonitrile. The elution was performed by a linear gradient of solvent B from 5% to 50% over 90 min. Peptides eluted from the column were manually collected, dried down, dissolved in 5% acetic acid, and analyzed by FAB/MS.

Mass Spectrometry. FAB mass spectra were recorded on a VG ZAB 2 SE double-focusing mass spectrometer, fitted with a VG cesium gun operating at 25 keV (2 μA). Samples were dissolved in 5% acetic acid and loaded onto a glycerol-coated probe tip; thioglycerol was added to the matrix just before introduction of the probe into the source. The amplification of the electrical signal was decreased during the magnet scan according to the intensity of the mass signals observed on the oscilloscope; mass spectra were recorded on UV-sensitive paper and manually counted.

Peptide Recognition. Signals recorded in the spectra were associated with the corresponding peptides on the basis of the expected molecular weight by using a computer program developed in our laboratory (Pucci & Sepe, 1988). A single Edman degradation step was performed on the whole peptide digests, followed by FAB/MS analysis of the truncated peptides in order to confirm the assignments as already described (Pucci et al., 1985).

Sequence Analysis. Automated Edman degradation was performed by using a Chelsea Instrument CI 4000 gas-phase sequencer equipped with an on-line Beckman System Gold HPLC, following the manufacturer's instructions (program Peptide I), on two different batch samples of the S-carboxymethylated protein.

Signal Peptide. Analyses for prediction of the cleavage site of the signal peptide were performed by using the software based on von Heijne's method (Von Heijne, 1986). Other protein sequence analyses were performed by using the University of Wisconsin's genetic computer group software package (Devereux et al., 1984).

Sequence Numbering. For the sake of simplicity, the sequence numbering of Morinaga et al. (1983) was followed. However, this numbering is incorrect since the Arg residue at position −1 following Morinaga et al. is the actual N-terminal amino acid (see Results).

Table I: Observed Mass Values in the FAB Spectrum of the Tryptic Digest of Human α-Fetoprotein[a]

position	MH^+	position	MH^+
47–49	377	292–313	2491
84–88	631	314–318	607
137–149	1661	329–341	1582
143–149	911	342–353	1388
150–165	1924	343–353	1232
151–165	1768	357–364	979
176–186	1297	385–394	1150
187–192	590	396–402	840
193–195	417	403–418	1892
196–209	1588	419–433	1645
210–214	594	420–433	1517
215–223	1008	450–468	2053
224–230	851	517–530	1563
239–252	1746	531–539	1103
253–264	1349	540–562	2585
265–279	1788	580–583	460
286–291	732	586–590	430

[a] Nominal mass values are listed.

Results

Direct Mass Spectrometric Analysis of Proteolytic Digests of α-Fetoprotein. Reduced and carboxymethylated α-fetoprotein was digested with trypsin and V-8 protease either separately on two different samples or combined on the same one. Three different peptide mixtures were generated and directly submitted to mass measurement without any purification steps.

Table I shows the results obtained from the analysis of the tryptic peptides; each signal recorded in the spectra was associated with the corresponding peptide along the α-fetoprotein amino acid sequence on the basis of its mass value and the enzyme specifity, following the FAB-mapping procedure. A computer program was used to locate mass values within the protein sequence (Pucci & Sepe, 1988). All the assignments were confirmed by submitting a portion of the digest to a single step of Edman degradation followed by mass analysis of the truncated peptides. With this one experiment it was possible to map 50% of the entire α-fetoprotein primary structure. Moreover, the tryptic map allowed the identification of the C terminus of the protein; the signal at m/z 430, in fact, corresponds to the C-terminal peptide 586–590, Ala-Ala-Leu-Gly-Val, thus confirming both the correctness of the reading frame and the positioning of the stop codon. This evidence was supported by data obtained from the V-8 protease digest of α-fetoprotein showing the occurrence of the expected C-terminal fragment 577–590 (see Table II). Furthermore, no shortened C-terminal proteolytic fragments were detected, suggesting that no heterogeneity occurred at the C terminus. It should be emphasized that the analysis of the C terminus is a very important piece of information both in the verification of protein sequences deduced from DNA sequences and in the characterization of DNA-recombinant proteins; this analysis is often a difficult task to accomplish with classical methods. In fact, previous reports from carboxypeptidase (Cpase) digestion analysis (Aoyagi et al., 1977) described a Gly-Ala-(Leu, Ala)-Val sequence as the C terminus of human α-fe-

Table II: Observed Mass Values in the FAB Spectrum of the V-8 Protease Digest of Human α-Fetoprotein[a]

position	MH+	position	MH+
7–14	851	339–360	2569
22–26	518	361–376	1967
51–69	2006	382–388	921
70–78	1060	382–405	2795
83–87	631	389–405	1893
138–146	1192	406–427	2534
182–193	1384	428–447	2228
194–196	417	448–455	906
236–242	828	456–470	1675
243–248	691	501–517	2026
249–263	1836	518–532	1705
264–282	2259	533–547	1799
283–290	1026	533–550	2169
291–299	1084	551–555	488
306–319	1573	556–561	665
306–327	2469	562–570	1052
328–338	1304	577–590	1441

[a] Nominal mass values are listed.

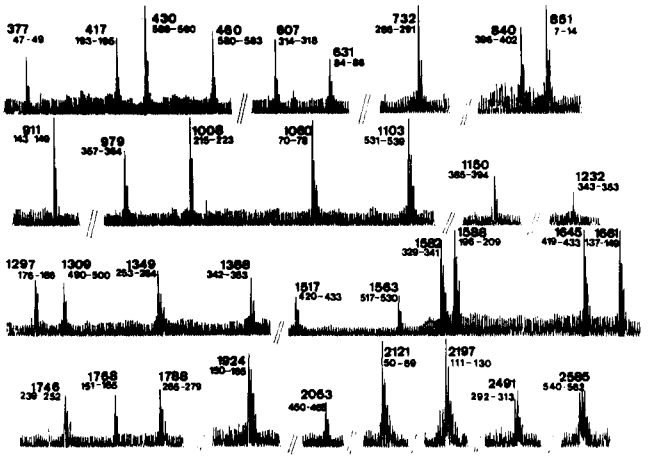

FIGURE 1: FAB/MS spectrum of human α-fetoprotein double digested with trypsin and V-8 protease. Nominal mass values are indicated. The relative abundances of the mass signals are on an arbitrary scale since the gain was varied during the scan.

toprotein. This sequence is representative of the difficulty in correlating Cpase digestion analysis and the actual protein sequence, since two consecutive identical residues are present, together with a glycine whose liberation rate cannot be easily correlated with its position in the sequence (Ambler, 1967).

As shown in Table I, most of the observed peptides were generated by the central or the C-terminal region of α-fetoprotein, the N terminus remaining largely uncovered. The tryptic digest was then submitted to a further enzymatic hydrolysis by V-8 protease. In fact, since the failure in recording all the α-fetoprotein tryptic peptides is due to the well-known suppression phenomena occurring in FAB analysis of peptide mixtures (Naylor et al., 1986), changing the physical-chemical properties of the peptides with a second digestion might have resulted in the mapping of those regions of the protein sequence unidentified in the tryptic map.

The double-digested protein was analyzed by FAB/MS, and the resulting spectrum is shown in Figure 1. As expected, a number of peptides corresponding to previously unidentified regions of the α-fetoprotein sequence were found. In particular, the signals at m/z 851, 2121, 1060, 502, and 2197, corresponding to peptides 7–14, 50–69, 70–78, 85–88, and 111–130, respectively, all originated from the N-terminal region of the protein.

With these two experiments, 63% of the α-fetoprotein sequence was verified and controlled; however, the N terminus of the protein was still elusive.

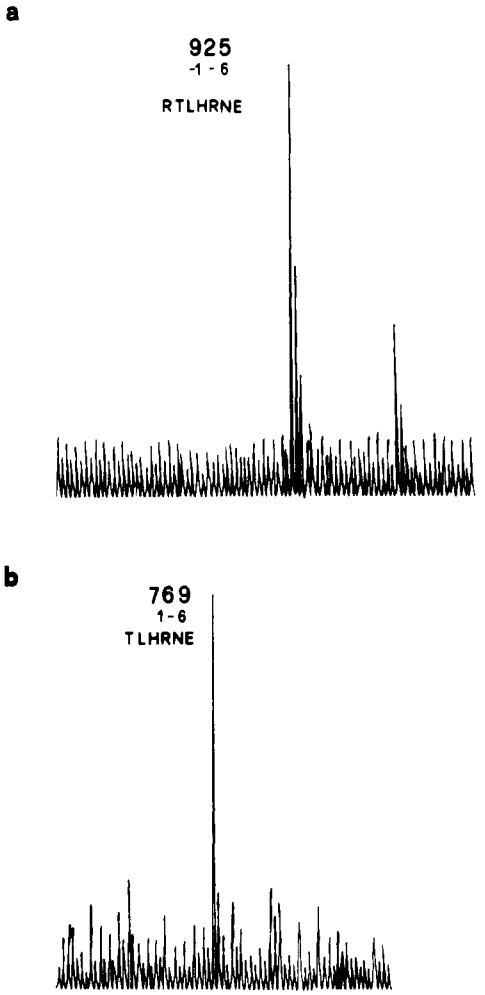

FIGURE 2: FAB/MS analysis of fraction 7 from the HPLC separation of V-8 digested α-fetoprotein (a) before and (b) after a single Edman degradation step. For the sake of simplicity, the Morinaga et al. (1983) numbering is reported.

A second sample of reduced and carboxymethylated α-fetoprotein was then hydrolyzed with V-8 protease; a portion of the digest was submitted to mass measurement without fractionating steps. The resulting V-8 protease map is shown in Table II; several previously unobserved peptides were identified in the mass spectrum, allowing a further verification of the α-fetoprotein sequence.

The combination of the FAB data obtained from the analysis of these three proteolytic digests of α-fetoprotein eventually resulted in the verification of more than 80% of its amino acid sequence. No information was obtained on the N-terminal peptide, thus leaving still uncertain both the nature of the first amino acid and the actual maturation site of the protein.

Analysis of the V-8 Protease Digest of α-Fetoprotein by HPLC and FAB/MS. In order to examine the N-terminal peptide of α-fetoprotein, a portion of the V-8 protease digest was fractionated by HPLC on a C-18 reverse-phase column. The fractions were manually collected, freeze-dried, and analyzed by FAB/MS; the resulting map accounts for about 64% of the entire α-fetoprotein sequence. Moreover, as shown in Figure 2a, the mass analysis of fraction 7 showed the occurrence of a signal at m/z 925 that could not be assigned to any fragment in the mature α-fetoprotein sequence according to Morinaga et al. This mass value was tentatively identified as corresponding to peptide 1–6, carrying an extra arginine residue at the N-terminus, i.e., peptide −1 to 6 with respect

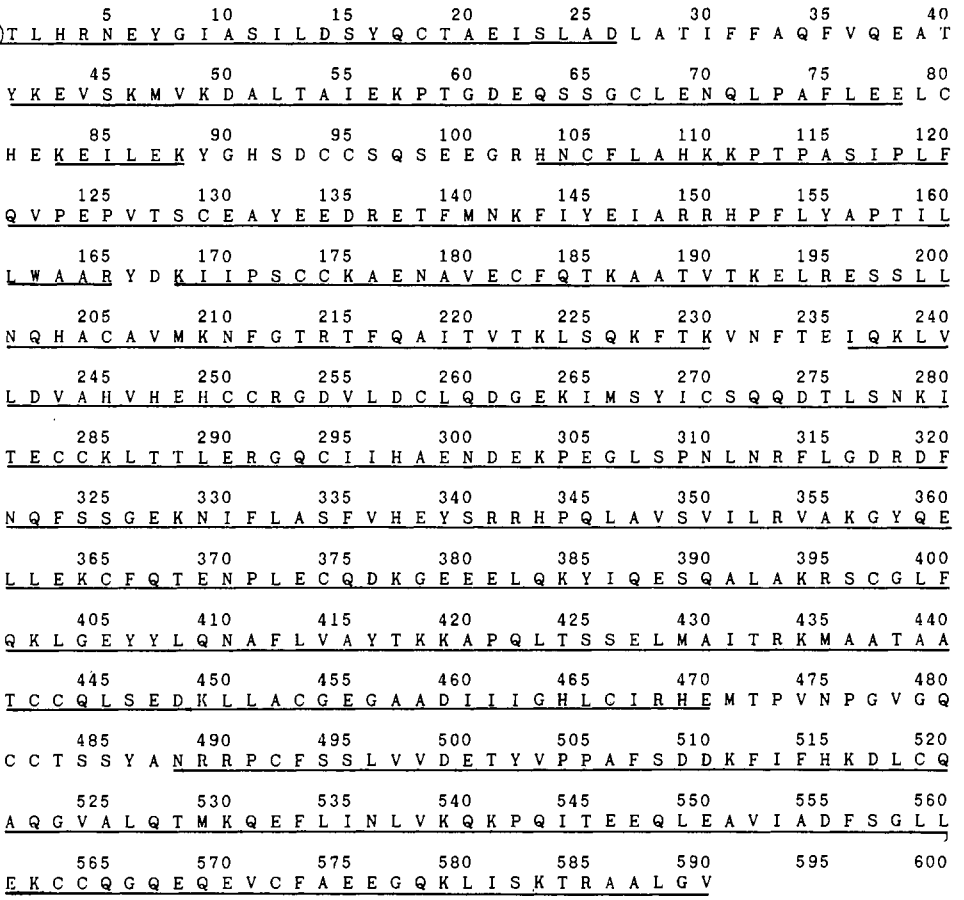

FIGURE 3: Primary structure of mature human α-fetoprotein from Morinaga et al. (1983); the amino acid sequence confirmed by FAB/MS analysis is underlined. Contrary to the previous report, an extra Arg is added to the N terminus; for the sake of simplicity, the Morinaga et al. (1983) numbering has been retained.

to the protein sequence of Morinaga et al. This assignment was easily confirmed by submitting fraction 7 to a single step of Edman degradation followed by FAB analysis of the truncated peptide. The mass signal was shifted back to m/z 769 by the loss of 156 mass units, thus indicating the presence of an arginine residue at the N-terminal position (Figure 2b).

The results obtained by combining the data from the FAB/MS analysis of both the unfractionated digests and the HPLC fractions from the V-8 protease hydrolysis of α-fetoprotein are summarized in Figure 3. The results led to the verification and the control of more than 90% of the entire primary structure of the protein, including the elucidation of the N-terminal amino acid, which was incorrectly predicted by Morinaga et al. (1983) on the basis of the results obtained by Ayoagi et al. (1977).

In order to be confident of the latter assignment, a reduced and carboxymethylated α-fetoprotein sample was submitted to 10 cycles of Edman degradation by using a gas-phase sequencer equipped on-line with an HPLC for PTH amino acid analysis (Pucci et al., 1983). Although a very low initial yield was observed (15%), the following unique sequence was inferred:

Arg–Thr–Leu–His–Arg–Asn–Glu–Tyr–Gly–Ile
(29) (82) (84) (29) (39) (63) (77) (57) (53)(48)

where the figures in parenthesis refer to picomoles of PTH amino acids recovered at each cycle.

Discussion

FAB/MS methodology was extensively used to verify the primary structure of α-fetoprotein produced by mass cell culture. The primary structure of over 90% of the protein, including both the amino and carboxy termini, was obtained by using this analytical approach (Figure 3). The results reported here validate the conclusions based on the analysis of the carbohydrate moiety (Terrana et al., 1990) that this α-fetoprotein preparation is highly homogeneous, since we showed that this preparation contains a single protein whose sequence is essentially identical with that derived from α-fetoprotein cDNA, but for the addition of Arg at the N terminus.

Identification of the N-terminal peptide required HPLC fractionation of the V-8 digest before FAB/MS analysis. This analysis revealed that arginine is the N-terminal amino acid of this peptide and consequently of the mature protein, rather than threonine as previously suggested (Morinaga et al., 1983; Gibbs et al., 1987). It is worth noting that previous studies on this protein by Aoyagi et al. (1977) and Yachnin et al. (1977) reported the following N-terminal sequences, respectively: (Ser)-Thr-Leu-His-Arg-Asn-Glu-Tyr-Gly-Ile-Ala-(Ser)-Ala-Leu-Asp-(Ser)-Tyr-Gln-(Cys)-(Ala) and Ser-Thr-Leu-His-Ser-Asn-Glu-Tyr-Gly-Ile-Ala-Ser-Ile-Leu-Asx-Ser-Tyr (residues in parenthesis were reported by the authors to be doubtful, those underlined are incorrect). Both sequences were mostly correct, except for the assignment at position 1. It is interesting to note that Yachnin et al. (1977) inferred the same amino acid residue to be at position 1 and at position 5, but at the time when the above studies were carried out, HPLC analysis of PTH amino acids was not in use, and indeed the analysis of PTH–Arg was a difficult task to achieve (Niall et al., 1973).

The conclusion that arginine is the N-terminal residue is confirmed by direct Edman degradation of the intact protein.

α-Fetoprotein

	-18 -10 +1 10 20
Human	MKWVESIFLIFLLNFTES RTLHRNEYGIASILDSYQCTAEISL
Rat	MKQPAT...SA..SFLL....A.P .V..T..F..E.T...S..PT.KNM
Mouse	...ITPAS..L..H.AA. KA..E..F....T...S..VT.KNV

Albumin

	-18 -10 +1 10 20
Human	....TF.S.L..FSSAY. .GVF.RDAHKSEVAHRFKDLG.ENF
Rat	....TFLL.L.ISGSAF. .GVF.R.AHKSE.AHRFKDLG.QHF

FIGURE 4: Revised alignment of human α-fetoprotein signal peptide and N-terminal sequence with the mouse and rat α-fetoprotein and with the homologous albumin from human and rat. Dots represent amino acids identical with those occurring in the corresponding positions of human α-fetoprotein.

Furthermore, inspection of the amino acid sequence derived from the cDNA sequence shows that threonine is preceded by arginine. Thus, these results can be easily reconciled with the cDNA data if one assumes that processing of the preprotein occurs by cleavage of the leader peptide between serine and arginine rather than between arginine and threonine as proposed by Morinaga et al. (1983) and Gibbs et al. (1987). The placement of the signal sequence cleavage site, as deduced from our structural data, agrees well with von Heijne's rules, which predict either Ala, Ser, Gly, Cys, Thr, Arg, or Glu at position −1 and any amino acid other than aromatic, charged, and polar at position −3 (von Heijne, 1986). In fact, the human α-fetoprotein sequence reported by Morinaga et al. was the only protein having an arginine residue at position −1 among 275 signal sequences in the compilation made by Watson (1984), whereas arginine at the N terminus was found in 15 proteins.

Inspection of the mouse α-fetoprotein sequence suggests that a similar correction should be made for this protein, which according to Law et al. (1981) has Ala-Ser-Lys-Ala at positions −3, −2, −1, +1, respectively, in disagreement with von Heijne's rule. We suggest that the correct end of the signal peptide should be Ser and the mouse α-fetoprotein N-terminal amino acid should be Lys rather than Ala. Mouse α-fetoprotein, in fact, was found to be the only protein possessing a lysine residue at position −1 when the compilation made by Watson (1984) was screened, whereas Lys occurred 19 times at position +1.

In the case of rat α-fetoprotein, the cleavage site of the putative signal peptide has been reported to be between a proline and an arginine residue (Jagodzinski et al., 1981; Turcotte et al., 1985). Aligning the α-fetoprotein N-terminal sequences as shown in Figure 4 reveals that the N-terminal sequence of human α-fetoprotein is identical with both rat and mouse α-fetoproteins at 13 of 25 positions, while the two rodent proteins share 19 of 25 amino acids. Such a high degree of identity could not have been so evident if the three proteins were aligned by the previously proposed NH_2 termini.

α-Fetoprotein and albumin share a high degree of identity throughout the molecule except in the amino-terminal region. A comparison of the human and rat albumin sequences (Figure 4) deduced from their respective cDNA's (Sargent et al., 1981; Dugaiczyck et al., 1982) shows that in both cases an 18 amino acid leader sequence has been identified, in agreement with direct analysis (Strauss et al., 1977; Russel & Geller, 1975; McConn et al., 1975). The similarity between albumin and α-fetoprotein became apparent from the very first studies, and it has led to the hypothesis that the two proteins are derived from the same ancestral gene. Evolution has introduced some structural differences in the two proteins: (1) α-fetoprotein is glycosylated while albumin is not; (2) albumin undergoes further processing to remove a six amino acid long propeptide after an Arg-Arg sequence while α-fetoprotein does not. The present results show that the site of cleavage of the signal sequence has been conserved during divergence of the two genes, since hydrolysis occurs at the same position from the amino terminus at the Ser-Arg pair in three out of five proteins (Figure 4). It is worth considering that in the other two proteins conservative substitutions have occurred. This extends the similarity between proalbumin and α-fetoprotein, since both proteins are composed of 591 amino acids after removal of the signal peptides, although proalbumin is further processed to remove the propeptide.

Nevertheless, although it seems evident that α-fetoprotein is secreted as a 591 amino acid protein, it cannot be excluded that the serum-circulating protein is then subjected to further posttranslational events. If so, the results from Ayoagi et al. (1977), obtained from a protein produced in vivo, could be explained by considering that the circulating α-fetoprotein might have been subjected to further removals of amino acid(s) from the N and/or C terminus.

In conclusion, all the analyses so far performed, i.e., immunological (Tecce & Terrana, 1988), carbohydrate (Terrana et al., 1990), and sequencing (present work), on this α-fetoprotein obtained from an industrial-size cell culture confirm that this preparation is highly homogeneous and consists of authentic α-fetoprotein. This and the fact that the protein comes from a constant source make it ideal to serve as a standard for calibration. An additional result came from the completion of the structural analysis presented above, showing that mature α-fetoprotein consists of 591 amino acid residues rather than 590, with arginine rather than threonine (Morinaga et al., 1983) at the N terminus, and that consequently the leader sequence peptide is made up of 18 rather than 19 amino acid residues.

Registry No. α-Fetoprotein (human HepG2 cell reduced), 133164-86-0; α-fetoprotein precursor (human HepG2 cell reduced), 133164-85-9.

References

Abelev, G. I. (1983) *Oncodev. Biol. Med. 4*, 371–381.

Ambler, R. P. (1967) *Methods Enzymol. 11*, 155–173.

Aoyagi, Y., Ikenaka, T., & Ichida, F. (1977) *Cancer Res. 37*, 3663–3667.

Biemann, K. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., Ed.) pp 279–306, Humana Press, Clifton NJ.

Devereux, S., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387–395.

Dugaczyk, A., Law., A. W., & Dennison, O. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 71-75.
Gibbs, P. E. M., Ziclinski, R., Boyd, C., & Dugaiczyk, A. (1987) *Biochemistry 26*, 1332-1343.
Gibson, B. W., & Biemann, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1956-1960.
Jagodzinski, L. L., Sargent, D. T., Yang, M., Glackin, C., & Bonner, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3521-3525.
Law, W. S., & Dugaiczyk, A. (1981) *Nature 291*, 201-205.
Meloun, B., Moravek, L., & Kostka, V. (1975) *FEBS Lett. 58*, 134-137.
Morinaga, T., Sakai, M., Wegman, T. G., & Tamaoki, T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4604-4608.
Morris, H. R., & Greer, F. M. (1988) *Trends Biotechnol. 6*, 140-147.
Morris, H. R., Panico, M., & Taylor, G. W. (1983) *Biochem. Biophys. Res. Commun. 117*, 299-305.
Naylor, S., Fiendeis, A., Gibson, B. W., & Williams, D. H. (1986) *J. Am. Chem. Soc. 108*, 6357-6363.
Niall, H. D. (1973) *Methods Enzymol. 27*, 942-1010.
Pucci, P., & Sepe, C. (1988) *Biomed. Environ. Mass Spectrom. 17*, 287-291.
Pucci, P., Sannia, G., & Marino, G. (1983) *J. Chromatogr. 270*, 371-377.
Pucci, P., Carestia, C., Fioretti, G., Mastrobuoni, A. M., & Pagano L. (1985) *Biochem. Biophys. Res. Commun. 130*, 84-90.
Rhodes, C. J., Brennam, S. O., & Hutton, J. C. (1989) *J. Biol. Chem. 264*, 14240-14245.
Russel, J. H., & Geller, D. M. (1975) *J. Biol. Chem. 250*, 3409-3413.
Sargent, T. D., Yang, M., & Bonner, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 243-246.
Sell, S. (1982) in *Human Cancer Markers* (Sells, S., & Wahren, B., Eds.) pp 133-164, Humana Press, Clifton, NJ.
Takao, T., Hitouji, T., Shimonishi, Y., Tanabe, T., Inouye, S., & Inouye, M. (1984) *J. Biol. Chem. 259*, 6105-6109.
Tecce, M. F., & Terrana, B. (1988) *Anal. Biochem. 169*, 306-311.
Terrana, B., Tecce, M. F., Manetti, R., Ceccarini, C., Lamba, D., & Segre, A. L. (1990) *Clinical Chem. 36*, 879-882.
Turcotte, B., Wertin, M., Cheverette, M., & Belanger, L. (1985) *Nucleic Acids Res. 13*, 2387-2398.
Urano, Y., Sakai, M., Watanabe, K., & Tanaoki, T. (1984) *Gene 32*, 255-261.
Von Heijne, G. (1986) *Nucleic Acids Res. 14*, 4683-4690.
Watson, M. E. E. (1984) *Nucleic Acids Res. 12*, 5145-5164.
Yachnin, S., Hsu, R., Heinrikson, R. L., & Miller, J. B. (1977) *Biochim. Biophys. Acta 493*, 418-428.

Real-Time Analysis of the Assembly of Ligand, Receptor, and G Protein by Quantitative Fluorescence Flow Cytometry†

Shawn P. Fay, Richard G. Posner, William N. Swann, and Larry A. Sklar*

Cytometry, University of New Mexico, School of Medicine, Albuquerque, New Mexico 87131, and National Flow Cytometry Resource, M888 Los Alamos National Laboratory, Los Alamos, New Mexico 87545

Received August 31, 1990; Revised Manuscript Received February 8, 1991

ABSTRACT: We describe a general approach for the quantitative analysis of the interaction among fluorescent peptide ligands (L), receptors (R), and G proteins (G) using fluorescence flow cytometry. The scheme depends upon the use of commercially available fluorescent microbeads as standards to calibrate the concentration of fluorescent peptides in solution and the receptor number on cells in suspension. We have characterized a family of fluoresceinated formyl peptides and analyzed both steady-state and dynamic aspects of ligand formyl peptide–receptor interactions in digitonin-permeabilized human neutrophils. Detailed receptor-binding studies were performed with the pentapeptide *N*-formyl-Met-Leu-Phe-Phe-Lys-fluorescein. Equilibrium studies showed that GTP[S] caused a loss of binding affinity of approximately two orders of magnitude, from ~0.04 nM (LRG) to ~3 nM (LR), respectively. Kinetic studies revealed that this change in affinity was principally due to an increase in the dissociation rate constants from $\sim 1 \times 10^{-3}$ s^{-1} (LRG) to $\sim 1 \times 10^{-1}$ s^{-1} (LR). In contrast, the association rate constants in the presence and absence of guanine nucleotide ($\sim 3 \times 10^{7}$ s^{-1} M^{-1}) were statistically indistinguishable and close to the diffusion limit. In the presence of guanine nucleotide (LR), the kinetic data were adequately fit by a single-step reversible-binding model. In the absence of guanine nucleotides, not all receptors have rapid access to G to form the LRG ternary complex. Mathematically, those R that have rapid access to G are either precoupled to R or the association of G with R is fast compared to the association of L with R. The physiological consequences of coupling heterogeneity are discussed.

Cellular signal transduction proceeds through a sequence of events that is initiated via an extracellular ligand–receptor interaction. In neutrophils, activation involves the interaction of ligand–receptor (LR) complexes with guanine nucleotide binding proteins (G) (Okajima et al., 1985; Lad et al., 1985). Rapid interconversions among at least three distinct states of the receptor, which have been described as LR, LRG, and LRX (a desensitized form of the receptor), have been observed (Sklar et al., 1989). In the human neutrophil, the interactions of the formyl peptide receptor with its G proteins are reversible (Okajima et al., 1985; Sklar et al., 1987), but no systematic

†This work was supported by NIH Grants AII9032 and RR01315.
*To whom correspondence should be addressed.

0006-2960/91/0430-5066$02.50/0