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Isolation and Partial Structural Characterization of an Equine Fibrinogen CNBr Fragment That Exhibits Immunologic Cross-Reactivity with an Aα-Chain Cross-Linking Region of Human Fibrinogen†

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ABSTRACT: Immunochemical studies of equine fibrinogen were conducted to characterize the structural basis for the immunologic cross-reactivity observed between human and equine Aα chains when employing an antiserum to the 26K, human cyanogen bromide (CNBr) fragment, Aα 241–476 (CNBr VIII). A 38K, equine CNBr fragment that reacts with this antiserum was isolated from CNBr-digested equine fibrinogen by Sephadex G-100 gel filtration. It was further purified by sequential hydrophobic chromatography on phenyl-Sepharose CL-4B, followed by reversed-phased (C-8) high-performance liquid chromatography (HPLC). NH_2-Terminal analysis of the purified fragment, designated EqAαCNBr, identified one major sequence whose first three residues, E-L-E, were identical with those of human CNBr VIII. Tryptic and staphylococcal protease digests of the equine fragment were resolved by reversed-phase HPLC (C-4, C-18), and the separated components were characterized by amino acid analysis and automated Edman degradation. A total of 34 tryptic and 20 staph protease peptides yielded sequence information that permitted the alignment of 271 equine residues with residues Aα 241–517 from the COOH-terminal two-thirds of the human Aα chain so that 63% of the possible matches were identical. Other features of interest included (1) an amino acid substitution in which the methionine residue at Aα 476 in the human Aα chain was replaced by a valine residue, thus accounting, in part, for the larger EqAαCNBr fragment obtained from the equine molecule, and (2) a region of striking homology in which 36 successive residues, corresponding to Aα 428–464 in the human Aα chain, were identical in both species. These findings, together with available structural data for the COOH-terminal portion of the rat and bovine Aα chains, indicate that the region corresponding to (human) Aα 240–517 represents a conserved portion of the fibrinogen molecule. This may, in turn, explain the difficulties encountered when trying to raise monoclonal antibodies to cross-linking regions that are contained within the COOH-terminal two-thirds of the human Aα chain.

The COOH-terminal portion of the Aα chain of human fibrinogen contains several structural domains that function to maintain hemostasis by mediating the interaction between fibrin(ogen) and a variety of plasma proteins and cellular components. Included among these domains are factor $XIII_a$ cross-linking regions that foster the covalent interaction between α chains of neighboring fibrin molecules (Doolittle et al., 1977; Fretto & McKee, 1978; Sobel et al., 1983), between α chains and α_2-antiplasmin (Sakata & Aoki, 1980; Tamaki & Aoki, 1982), and between α chains and fibronectin (Mosher, 1975; Sobel et al., 1983). Plasmin-sensitive cleavage sites are also clustered within the COOH-terminal two-thirds of the Aα chain (Pizzo et al., 1970; Takagi & Doolittle, 1975), and an RGDS platelet recognition site has been localized to this region as well (Gartner & Bennett, 1985; Hawiger et al., 1989).

While these various interactions provide a role for the COOH-terminal portion of the Aα chain in maintaining hemostasis, the mechanisms by which this is achieved, in most cases, remain poorly defined. As one approach toward elu-

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cidating some of the structure–function relationships that exist within this part of the fibrinogen molecule, anti-COOH Aα-chain monoclonal antibodies have been successfully employed as structural probes in studies designed to learn more about the processes of α-polymer formation (Sobel et al., 1988a,b) and Aα-chain degradation (Liu et al., 1986). In another application, as yet unrealized, monoclonal antibodies that are fibrin-specific because their determinant includes an α–α-chain cross-link could serve as diagnostic reagents for the detection of thrombosis.

In the course of trying to develop murine hybridoma cell lines to various antigens from the COOH-terminal two-thirds of the human (A)α chain (i.e., the region that spans residues Aα 208–625, represented by the CNBr peptides, CNBr V–XI,[1] we made several observations which suggest that a large repertoire of antibodies to this functionally critical region may not be readily obtained using traditional hybridoma techniques. First, while three different derivatives from the region, Aα 241–584, each produced a strong immune response (based on the high serum antibody titers observed in the immunized mice), isolating the anti-Aα-chain immunoglobulins from the resulting hybridoma fusions, which were cultured in the presence of either fetal calf or horse serum, proved to be consistently difficult. Second, only two cell lines that secrete anti-human COOH-terminal Aα-chain antibodies were successfully obtained from cultures containing fetal calf serum, and neither of these antibodies recognized bovine fibrinogen, when examined by radioimmunoassay (Ehrlich et al., 1983). In at least one case, this lack of cross-reactivity could be explained by the fact that the antibody recognized an epitope in the human Aα chain that was missing from the structure of the bovine molecule, based on available sequence data (Lottspeich & Henschen, 1978; Watt et al., 1979; Henschen et al., 1980; Chung et al., 1982). Finally, sera from mice immunized with the peptide Aα 290–347 were found to react, in immunoblotting studies, with several components present in horse serum utilized as a tissue culture additive. No immunoreactivity was observed when control, nonimmune mouse sera were employed as the primary antiserum.

Collectively, these immunologic observations suggest that various animal sera that are used as supplements for tissue culture media contain residual fibrin(ogen) degradation products whose structures are similar to regions from the COOH-terminal two-thirds of the human Aα chain. If so, then the inadvertant introduction of these fragments into hybridoma cultures secreting anti-COOH-Aα-chain antibodies could actually mask the presence of immunoglobulins of interest by competing with the screening antigen for antibody binding. To data, there is no structural information available for the region of equine fibrinogen that corresponds to the COOH-terminal two-thirds of the human Aα chain, and sequence data for this region in bovine fibrinogen are incomplete (Henschen et al., 1980; Chung et al., 1982).

In view of these considerations and because horse serum is increasingly favored for culture medium enrichment due to its lower cost, we undertook to isolate and characterize a CNBr fragment of equine fibrinogen, referred to here as EqAαCNBr, that by virtue of its immunologic cross-reactivity with human CNBr VIII was likely to be structurally homologous as well. Information about sequence similarities and differences between equine (or bovine) and human Aα chains, in this region of interest, could eventually be applied to optimize hybridoma protocols for the isolation of monoclonal antibodies to specified COOH-terminal Aα-chain determinants. The findings obtained here not only reveal extensive amino acid homology between human and equine Aα chains but also indicate, when compared with data reported for the rat (Crabtree et al., 1985) and bovine (Henschen et al., 1980; Chung et al., 1982) molecules, that the COOH-terminal two-thirds of the fibrinogen Aα chains is highly conserved.

Materials and Methods

All chemicals were reagent grade unless otherwise indicated. Additional methods appear in figure legends under Results.

Isolation and Purification of EqAαCNBr. Equine fibrinogen (82% clottable; Sigma, St. Louis, MO) was digested with cyanogen bromide (CNBr), and the resulting mixture of fragments was gel-filtered on Sephadex G-100 (Pharmacia, Piscataway, NJ) in 10% acetic acid as previously described for the isolation of human CNBr VIII (Sobel et al., 1982). Fragments of interest were further purified by hydrophobic chromatography on phenyl-Sepharose CL-4B (Pharmacia, Piscataway, NJ). The column was equilibrated in 1.7 M ammonium acetate, pH 3.1, and a linear gradient of increasing urea concentration (0–8 M) was used to elute the hydrophobic peptides. Material in the hydrophilic pool was further purified by reversed-phase HPLC (see below) on a semipreparative (10 mm × 25 cm) Dynamax C-8 column (Rainin, Woburn, MA). The mobile phase consisted of 0.1% TFA (buffer A) and 80% acetonitrile in 0.1% TFA (buffer B). The column was equilibrated in 20% B and developed with a linear gradient of increasing acetonitrile concentration to 45% B over a period of 50 min. Chromatography was conducted at room temperature at a flow rate of 5 mL/min.

HPLC Fingerprinting of EqAαCNBr. EqAαCNBr (Figure 5, pool II) was reduced with dithiothreitol and carboxymethylated using recrystallized [^{3}H]iodoacetic acid (Amersham, Arlington Heights, IL) according to methods described in several previous publications from this laboratory (Sobel et al., 1982, 1983). The specific activity of the [^{3}H]iodoacetic acid preparations used here ranged from 0.55×10^6 to 1.14×10^6 cpm/μmol. Trypsin (TPCK; Sigma, St. Louis, MO) digestion of reduced, alkylated EqAαCNBr was conducted in 0.2 M ammonium bicarbonate, pH 7.8, at a protein concentration of 0.1–0.2% and a final substrate:enzyme ratio of 50:1 (w/w). Trypsin was added in two equal aliquots, at T_0 and T_{45} (0 and 45 min), and the incubation was continued for a total of 2 h at 37 °C. *Staphylococcus aureus* protease (Sigma, St. Louis, MO) digestion was conducted in 0.1 M ammonium bicarbonate, pH 7.8, at a protein concentration of 0.1% and a final enzyme:substrate ratio of 40:1 (w/w). Digestion was allowed to proceed for 2 h at 37 °C and then continued for an additional 18 h at room temperature. Tryptic and staph protease digestions were interrupted by the addition of TFA (to 0.1%, final concentration), and the mixture of peptides was then concentrated by vacuum centrifugation prior to HPLC fingerprinting. Tryptic peptides were separated by reversed-phase HPLC on an analytical (0.46 × 25 cm) Vydac C-4 column (The Sep/a/ra/tions Group, Hesperia, CA). The mobile phase consisted of 0.1% TFA (buffer A) and 60% acetonitrile in buffer A (buffer B). The column was equilibrated in buffer A and developed with a linear gradient of

[1] Abbreviations: CNBr I–XI, 11 CNBr peptides of the human Aα chain, referred to by Roman numeral according to their relative position from the NH_2-terminus of the molecule; EqAαCNBr, equine equivalent of the human Aα-chain region, CNBr VIII–IX–X; HPLC, high-performance liquid chromatography; Staph protease, *Staphylococcus aureus* protease; TFA, trifluoroacetic acid; SCMC, *S*-(carboxymethyl)cysteine; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IAA, iodoacetic acid.

Table I: NH_2-Terminal Sequence of the 78K Equine Fibrinogen Chain (See Figure 1)[a]

	cycle													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
78K band	X	E	E	G	E	F	L	H	E	G	G	G	V	R
	–	29.1	28.2	21.5	24.1	13.1	21.8	–	22.4	27.3	31.6	34.8	10.5	–
horse, mule 1 FPA[b]	T	E	E	G	E	F	L	H	E	G	G	G	V	R

[a] Equine fibrinogen (30 μg) was subjected to SDS–PAGE (10% gels, reducing conditions), and the components were transferred to PVDF membranes for microsequencing, as described in the text. Data are expressed as picomoles of PTH-amino acid released per step, with residues represented in single-letter code. [b] Expected sequence of equine FPA as reported in Dayhoff et al. (1972).

increasing acetonitrile concentration to 75% B over a period of 150 min. Chromatography was conducted at 42 °C at a flow rate of 1 mL/min. In some cases, peptides were rechromatographed on an analytical (3.9 mm × 30 cm) μBondapak C-18 column (Waters, New Milford, MA) for improved resolution. The mobile phase consisted of 0.05% TFA (buffer A) and 60% acetonitrile in buffer A (buffer B). The column was equilibrated in buffer A and developed, after 5 min at initial conditions, with a linear gradient of acetonitrile concentration increasing at a rate of 0.5% B/min. Chromatography was conducted at room temperature at a flow rate of 1 mL/min. Staph protease peptides were separated on an analytic (0.46 × 25 cm) Vydac C-18 column, using a mobile phase comprised of 0.1% TFA (buffer A) and 80% acetonitrile in buffer A (buffer B). Chromatographic conditions were as described above for the initial separation of equine tryptic peptides. Where indicated, staph protease peptides were further resolved by rechromatography (C-18) under shallower gradient (0.125% B/min), or isocratic, elution conditions. Fractions from both the tryptic and staph protease fingerprints were concentrated by vacuum centrifugation prior to subsequent characterization by amino acid analysis and NH_2-terminal sequencing (see below). Peptides that contained cysteine [determined as [^{3}H](carboxymethyl)cysteine; ^{3}H-SCMC] were initially identified in each column effluent by counting aliquots of selected fractions in Scintiverse (Fisher, Springfield, NJ).

High-Performance Liquid Chromatography. HPLC-grade water (Fisher, Springfield, NJ), TFA (Pierce, Rockford, II), and acetonitrile (Baker, Phillipsburg, NJ) were employed for the preparation of all buffers. Absorbance was routinely monitored at both 214 and 280 nm. Chromatography was conducted on a Waters Associates System (Waters, New Milford, MA) equipped with a Model 730 data module, a Model M-45 solvent delivery system, a Model 481 variable-wavelength detector, and both a manual, Model U6K, and an automatic, Model 710A, injector. Data were also collected and processed on a Nelson Analytical Series Model 4400X chromatography data system (Hewlett Packard, Avondale, PA).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Discontinuous slab gel electrophoresis was conducted under nonreducing and/or reducing conditions as described by Laemmli (Laemmli & Favre, 1973). Homogeneous or gradient resolving gels, with a 4% stacking gel, were cast using reagents especially purified for electrophoresis (Bio-Rad, Richmond, CA). In some cases, SDS–PAGE was conducted on a PhastSystem (Pharmacia–LKB, Piscataway, NJ) employing either precast gradient or homogeneous gels. Prestained molecular weight standards were purchased as a mixture (Bethesda Research Laboratories, Gaithersburg, MD) and included on every run.

Immunoblotting. Electrophoresed components were transferred onto nitrocellulose (0.45 μm; Schleicher & Schuell, Keene, NH) as described by Towbin (Towbin et al., 1979) and the transfers processed according to reported methodology (Burnette, 1981). The rabbit antiserum, HS2-10, which was raised against human CNBr VIII (Sobel et al., 1982), was employed at dilutions of 1:1000–1:5000. Incubation with the primary antiserum was conducted for 4–18 h, either at room temperature or at 4 °C, depending on the antiserum dilution. Immunoreactive components on the transfer were detected following a 4-h incubation at room temperature with a 1:500 dilution of horseradish peroxidase conjugated swine anti-rabbit immunoglobulins (Dako; Accurate Biochemicals, Shamesway, NY) and subsequent rection with the substrate 4-chloro-1-naphthol (Bio-Rad, Richmond, CA), as specified by the manufacturer. Transfers to be analyzed for total protein, rather than for immunoreactivity, were stained with a 0.1% solution of Amido Black in 40% methanol/10% acetic acid and then destained in the same solvent.

Electroblotting onto Poly(vinylidene difluoride) Membranes (PVDF). Electrophoresed components were transferred onto PVDF membranes (0.45 μm; Millipore, Bedford, MA) and the transfers processed exactly as described by Matsudaira (Matsudaira et al., 1987). Bands of interest were excised and then subjected to microsequencing (see below).

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl for 24 h under vacuum at 110 °C in a Pico Tag workstation and then subjected to analysis on a Pico Tag amino acid analyzer (Waters, Milford, MA). Approximately 5–10% of the total material in selected HPLC fractions was analyzed.

NH_2-Terminal Sequencing. Automated Edman degradation was conducted on an Applied Biosystems (Foster City, CA) Model 470A sequencer equipped with an on-line Model 120A PTH analyzer. Generally, 25–80% of the total material in selected HPLC fractions was sequenced for at least 20 cycles. Microsequencing of proteins bound to PVDF membranes (see above) was conducted starting from an initial SDS–PAGE load of approximately 40–200 pmol.

Computer Analysis. The homology between equine and human Aα chains (in the region corresponding to human Aα 240–517) was evaluated by using the computer program Bestfit (Smith & Waterman, 1981), which is included in the Genetics Computer Group (GCG) sequence analysis package (Devereux et al., 1984). The percent of identically matched residues determined by computer analysis (64.5%, with five gaps) was very similar to the value derived for the alignment presented in Figure 8.

Results

Cross-Reactivity between Equine and Human COOH-Terminal Aα-Chain Regions. Figure 1, lane a, illustrates the Coomassie-stained SDS–PAGE pattern obtained when equine fibrinogen was electrophoresed under reducing conditions. The migration of the Aα, Bβ, and γ chains of human fibrinogen is shown in lane b, for comparison. Table I lists the microsequencing results obtained for a PVDF transfer of the 78K band of equine fibrinogen. These NH_2-terminal sequence data were consistent with the reported structure of equine fibrinopeptide A (Dayhoff et al., 1972) and served to identify the slowest of the three bands observed in Figure 1, lane a, as the equine Aα chain.

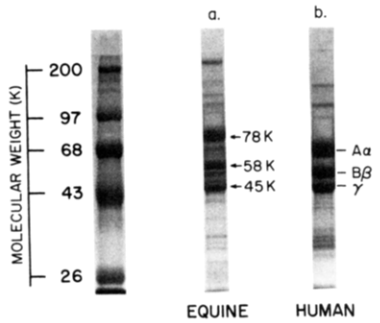

FIGURE 1: SDS–PAGE analysis of equine and human fibrinogens. Equine (lane a) and human (lane b) fibrinogens were subjected to SDS–PAGE on 8% gels under reducing conditions. Approximately 25 μg was applied to each lane. The gels were stained with Coomassie Brilliant Blue R-250 following electrophoresis. The positions of the Aα, Bβ, and γ chains of human fibrinogen are indicated, and the migration of standard molecular weight markers is included in the extreme left lane, for reference.

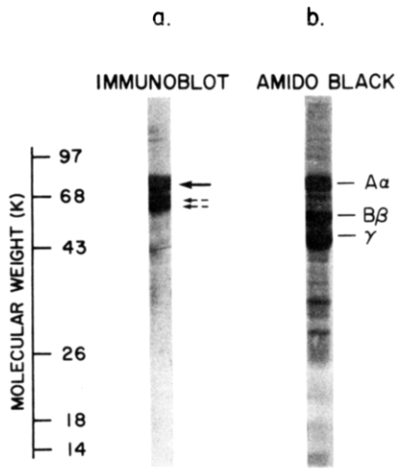

FIGURE 2: Cross-reactivity between equine and human Aα chains detected by Western blotting with HS2-10. Equine fibrinogen was subjected to SDS-PAGE on 10% gels under reducing conditions. Approximately 15 and 30 μg was applied to lanes a and b, respectively. The separated components were transferred to nitrocellulose and the transfers processed for immunoblotting (lane a) and total protein detection (lane b) as described in the text. HS2-10 was employed at a 1:5000 dilution. The positions of the equine Aα, Bβ, and τ chains are indicated in lane b. The arrows in lane a identify immunoreactive components. The migration of standard molecular weight markers is indicated at the left of the figure, for reference.

Figure 2, lane a, illustrates the immunoblotting findings obtained when equine fibrinogen was tested for its ability to react with HS2-10, a rabbit antiserum raised against human CNBr VIII. Lane b shows a duplicate transfer stained for total protein with amido black. As indicated by the solid arrow in lane a, a major band of immunoreactivity was localized to the 78K equine Aα chain. Immunoreactive bands of slightly lower molecular weight, suggestive of degraded Aα chains, were also observed, and their positions are indicated by the dashed arrows in lane a of Figure 2. None of these bands coincided with the Bβ and τ chains of equine fibrinogen, thus localizing the observed cross-reactivity with human CNBr VIII to the Aα chain of equine fibrinogen, exclusively.

Isolation and Characterization of EqAαCNBr. Figure 3 illustrates the results obtained when CNBr-treated equine fibrinogen was gel-filtered on Sephadex G-100 and the column effluent assayed for the elution of cross-reacting fragments by immunoblotting with the antiserum HS2-10. As shown in the inset of Figure 3A, the Coomassie-stained SDS–PAGE pattern indicated the partial resolution of fragments that ranged in molecular weight from 76K to less than 18K.

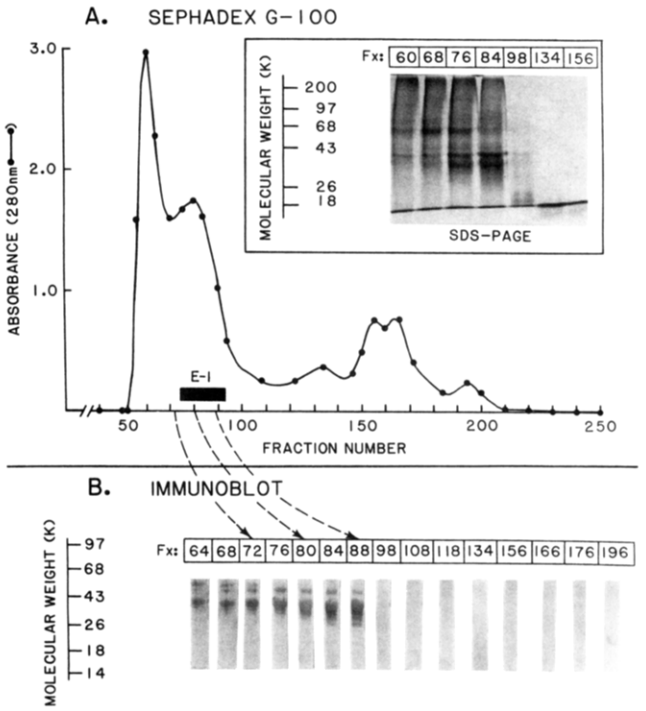

FIGURE 3: Isolation of EqAαCNBr by Sephadex G-100 gel filtration. (Panel A) CNBr-treated equine fibrinogen (625 mg) was applied to a column (4.0 × 149 cm) of Sephadex G-100 equilibrated in 10% acetic acid, and 9-mL fractions were collected. Fractions within the region labeled E-1 were pooled for further study. Inset: Approximately 0.10% of each indicated fraction was subjected to SDS–PAGE on 8% gels under nonreducing conditions. Coomassie Blue was employed for protein visualization. The migration of standard molecular weight markers is indicated at the extreme left of the inset. (Panel B) Approximately 0.10% of each indicated fraction was subjected to Western blotting using transfers from 5–20% SDS–PAGE gradient gels run under reducing conditions. HS2-10 was employed at a 1:1000 dilution. The migration of standard molecular weight markers is indicated at the extreme left of panel B, for reference.

Among these, the immunoblotting results, shown in Figure 3B, identified a heterogeneous population of immunoreactive peptides (27K-51K) in which species in the range of 30K-40K predominated. The same pattern was observed under nonreducing (not shown) and reducing conditions. The fractions indicated by the bar, labeled E-1 in Figure 3A, were pooled for further study as a source of EqAαCNBr.

Figure 4A illustrates the 280-nm absorbance profile obtained when the G-100 pool, E-1 (see Figure 3), was subjected to hydrophobic chromatography on phenyl-Sepharose CL-4B. Figure 4B shows the silver-stained SDS–PAGE patterns observed for selected fractions from the hydrophilic (left) and hyrophobic (right) regions of the column effluent. Most of the fragments contained within the G-100 pool, E-1 were relatively hydrophobic, based on the intensity and number of silver-stained bands observed for material that was eluted from phenyl-Sepharose (see Figure 4B, right). As shown in Figure 4C (right), none of these fragments, whose major representatives migrated with apparent molecular weights of 40K–50K, reacted with the antiserum HS2-10. Immunoreactivity was, however, observed exclusively among the few hydrophilic peptides (30K–40K) that did not bind to phenyl-Sepharose (see Figure 4B,C, left). The fractions indicated by the bar, labeled E-2 in Figure 4A, were pooled for further purification.

Figure 5 shows the 214-nm absorbance profile obtained when pool E-2 (see Figure 4A) was subjected to reversed-phase (C-8) HPLC. Two well-resolved peaks were observed, and these are labeled I and II in the figure. SDS–PAGE and immunoblotting analysis (see inset) indicated that the earlier

Table II: NH_2-Terminal Sequences of EqAαCNBr, Pools I and II (See Figure 5)[a]

	cycle															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
pool I	E	L	E	T	A	G	R	V	G	K	P	Q	V	D	P	V
	1.05	1.73	0.74	0.34	1.17	1.49	0.29	0.73	1.56	0.67	1.34	0.55	0.63	0.36	1.12	0.45
pool II	E	L	E	T	A	G	R	V	G	K	P	Q	V	D	P	V
	1.96	2.62	2.63	1.04	2.57	6.04	0.97	2.01	5.72	2.63	3.99	1.43	1.98	1.83	3.98	1.82

[a] Approximately 140 μg of pool I and 450 μg of pool II were subjected to automated Edman degradation as described in the text. Results are expressed as nanomoles of PTH-amino acid released per step. Values for PTH-Pro and -Gly (and -Ser) are not corrected for the high background observed for each of these residues at every step of the degradation.

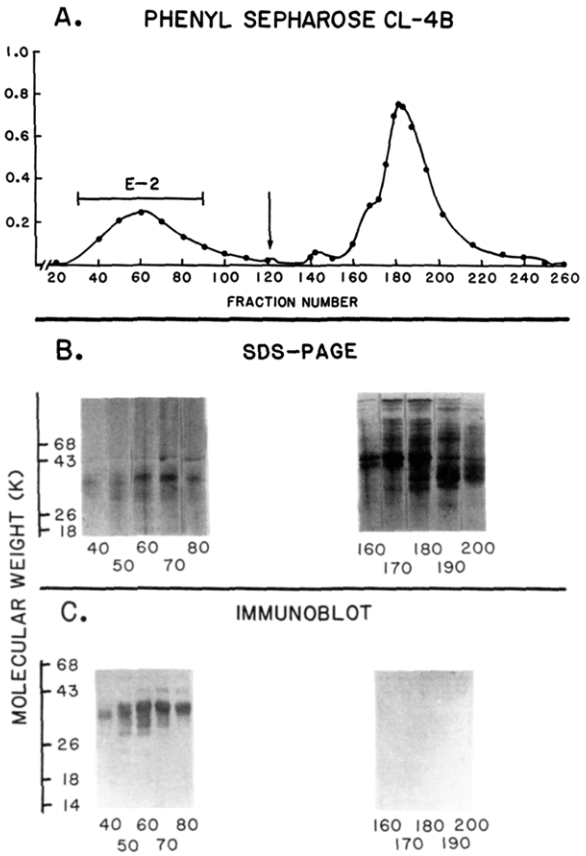

FIGURE 4: Purification of EqAαCNBr by phenyl-Sepharose CL-4B hydrophobic chromatography. (Panel A) Pool E-1 (see Figure 3), originating from 1.25 g of CNBr-treated equine fibrinogen, was applied to a column (2.5 × 40 cm) of phenyl-Sepharose CL-4B equilibrated in 1.7 M ammonium acetate, pH 3.1. Chromatography was conducted as described in the text, and 9-mL fractions were collected. The arrow indicates the start of elution conditions. Fractions within the region labeled E-2 were pooled for further study. (Panel B) Approximately 0.006% of each indicated fraction was subjected to SDS–PAGE on precast 10–15% gradient gels run under nonreducing conditions. The PhastSystem was employed, and the gels were silver-stained following electrophoresis. (Panel C) Approximately 0.10% of each indicated fraction was subjected to Western blotting exactly as described for panel B of Figure 3.

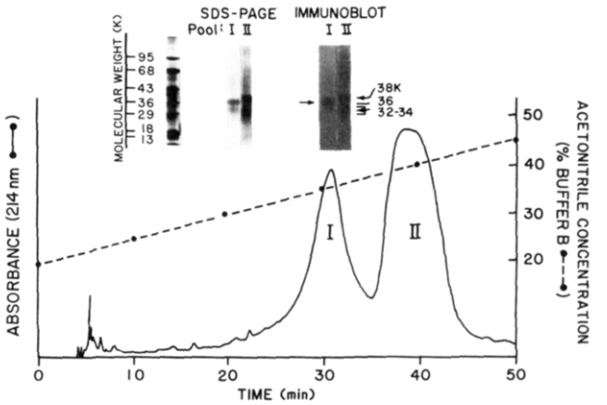

FIGURE 5: Purification of EqAαCNBr by C-8 reversed-phase HPLC. Approximately 8% of the total material recovered in pool E-2 (see Figure 4) was dissolved in 2.5 mL of buffer A and subjected to C-8 reversed-phase HPLC as described in the text. Five-milliliter fractions were collected. The dashed line indicates the acetonitrile gradient used for elution. The two pools obtained are labeled I and II. Inset: Approximately 170 ng of pool I and 470 ng of pool II were subjected to SDS–PAGE on precast 12.5% homogeneous gels run under reducing conditions using a PhastSystem. One gel was silver-stained, and a duplicate was processed for immunoblotting as described in the text. HS2-10 was used at a 1:5000 dilution. The apparent molecular weights of the various EqAαCNBr fragments are indicated, and the migration of standard molecular weight markers is shown in the extreme left lane, for reference.

eluting peak (pool I) contained one major immunoreactive fragment (36K). While the later eluting peak (pool II) exhibited some heterogeneity in size (32K–34K, 38K), each of the bands observed reacted with the antiserum HS2-10. The major component in pool II (38K) was the largest of all the fragments present in either pool.

Table II lists the quantitative sequence data obtained for the first 16 steps following automated Edman degradation of material from pools I and II. The same NH_2-terminal sequence, E-L-E-T-A..., was the major one identified in each case. These findings suggested that the various immunoreactive fragments, distributed between pools I and II (see Figure 5), shared a common NH_2-terminal structure but differed in the extent to which their COOH-terminal regions were preserved, reflecting the intrinsic heterogeneity of their parent Aα chains. Pool II was selected as the source of material for subsequent structural studies since, by virtue of its large size, the 38K fragment was considered to be the most "intact" representative of the equine equivalent of human CNBr VIII.

HPLC Fingerprinting. Figure 6A illustrates the reversed-phase HPLC elution profile obtained for a tryptic digest of EqAαCNBr. The peaks in the main portion of the figure are numbered sequentially according to their relative retention times, and only those which ultimately provided unique sequence information are labeled. The inset illustrates the 214-nm absorbance profile obtained when material from peak 1 was rechromatographed to partially resolve the tryptic peptides that failed to bind to the initial support. As indicated by the dashed lines and hatched areas in Figure 6A, two major peaks of radioactivity (representing the elution of cysteine-containing peptides) were localized, and together these accounted for 61% of the applied counts. (Additional peaks of radioactivity were also present, but these were minor, comprising less than 10% of the counts originally present in the load.)

Table III lists the 34 peptides (including free lysine and arginine) that were identified in the tryptic fingerprint, following compositional and NH_2-terminal sequence analysis of the material in each of the numbered peaks shown in Figure 6A. Comparison of these data with the reported primary structure of the human Aα chain (Lottspeich & Henschen,

Table III: EqAαCNBr Tryptic Peptides Isolated by Reversed-Phase HPLC (See Figure 6A)[a] and Their Apparent Homology to Equivalent Human Peptides

peak (Figure 6A)	equine peptide[b]	nmol recovered[c]	homologous human Aα-chain residue[d]
1A	(GD)K	8.3 (see peak 12-2)	419–421
1A	(S)R	6.8	
1A	R	8.9	440
1A	K	4.4	
1B	TQSM	12.5	
1C	SCSK	15.9	441–444
1D	AGSSGTGSSSK	13.5	
1E	EVTK	24.5	458–461
2-1	TVTK	6.0	445–448
2-2	EYHTGK[e]	3.8 (see peaks 3-1, 4)	408–413
3-1	EYHTSK	11.6 (see peaks 2-2, 4)	408–413
3-2	XSGSXQPTR	3.3	
4	KEYHTSK	7.2 (see peaks 2-2, 3)	407–413
5-1	LVTSK	18.9	414–418
5-2	VTSGSTTTTR	35.3	430–439
6-1	VRPDSSGHGNTR	6.8 (see peaks 17, 20)	374–385
6-2	ELETAGR	4.1	241–247
6-3	TSGSSGPGSASTR	*f* (see peaks 14, 16)	
7	HPGSSEPGSDGPR	24.4	290–302
8	TVIGPDGHK	26.8	449–457
9	HPGSSEPGSDGLQK	6.5	342–353
10	HPGSGSASTW	1.9	
11	PVPHGTGSVPESPR[g]	3.2 (see peak 13)	
12-1	ELLIGGEK	10.7	422–429
12-2	GDKELLIGGEK	1.7 (see peak 1A-1)	419–429
13	VGKPQVDPVPHGTGSVPESPR	3.2 (see peak 11)	
14	KPGSSGTLSASIWTSGSSGPGSASTR	7.1 (see peak 6-3, 16)	303–328
15	WIAGSSGTGSGSIW	1.5	
16	KPGSSGTLSASIW	9.5 (see peaks 6-3, 14)	303–315
17	VRPDSSGHGNTRPINPDWGTFEEVSGSVSPGTK	7.6 (see peaks 20, 6-1)	374–406
18	HPDEAAFFDSFSSK	15.7	494–508
19	TFPGEGSLDGLFHR	9.3	
20	PINPDWGTFEEVSGSVSPGTK	10.6 (see peaks 17, 6-1)	386–406
21	EVVNSEDGSDCGDAVELDLFR	10.1	462–482

[a] Material from the peaks labeled 1(A–E)–21 (see Figure 6A) was taken for sequencing and compositional analysis as described in the text. Peptide yield, in most cases, was 30–75%. Where intact and split products were both represented (see footnote *c*), recoveries were summed. Yields were calculated from compositional data obtained for each peptide and an HPLC load of 46.8 nmol, determined from radioactivity measurements and the finding of two cysteine-containing peptides within the structure of EqAαCNBr. [b] Data are presented in single-letter code. All the structures shown were derived from NH_2-terminal sequence data collected through each peptide's carboxyl terminus, except for the ones in peak 1A, which were inferred from compositional data alone. An extra glycine residue was also noted in the amino acid analyses of material from peak 1A. [c] Based on amino acid analysis. In several cases, incomplete tryptic cleavage or contaminating chymotryptic activity resulted in the release of multiple forms of a peptide. Their elution positions are identified, in parentheses, in column 3. [d] Based on data reported by Henschen et al. (1978, 1979), Lottspeich and Henschen (1978), Watt et al. (1979), and Kant et al. (1983). Homology was assigned only where visual inspection indicated obvious strong similarity between equine and human sequences. [e] Gly/Ser polymorphism (see peak 3) was noted at position 5 in this peptide. [f] This peptide was not recovered in the fingerprint shown in Figure 6A, but was isolated at 29% yield from another preparation of trypsin-treated EqAαCNBr run under similar conditions. [g] This anomalous cleavage product is consistent with acid-mediated cleavage at Asp/Pro (see peak 13).

1978; Henschen et al., 1978, 1979; Watt et al., 1979; Kant et al., 1983) suggested a strong similarity between many of the equine peptide sequences and sequences from the CNBr VIII and CNBr IX regions of the human Aα chain. These are listed in the last column of Table III. Sequence data for the major peptide recovered in peak 21 (following C-18 rechromatography) indicated that the methionine residue that defines the COOH terminus of human CNBr VIII was replaced by a valine residue in the equine molecule (see Table III; 15th residue in peptide 21). In addition, a single methionine-containing peptide, TQSM (see Table III; Peak 1B), was identified in the tryptic fingerprint shown in Figure 6A. These findings are consistent with the isolation of an equine CNBr fragment that was larger than its human counterpart, CNBr VIII (Aα 241–476), and included structural elements from within CNBr IX (Aα 477–517) as well.

Figure 6B illustrates the reversed-phase HPLC elution profile obtained for a staph protease digest of EqAαCNBr. Cysteine-containing peptides were distributed over several peaks, three of which accounted for the major proportion (64%) of the counts originally present in the load. Characterization of the fingerprint by compositional and NH_2-terminal sequence analysis identified a heterogeneous set of cleavage products. These apparently resulted from several incompletely cleaved glutamic acid residues, as well as from cleavages at a number of protease-sensitive, aspartic acid and (unexpectedly) glycine residues. Twenty unique staph protease peptides were localized within the peaks identified in Figure 6B (peaks that contributed "redundant" sequence information are not numbered), and their respective sequences are depicted schematically in Figure 7 (see S2-1 through S13-3).

Figure 7 shows the primary structure of EqAαCNBr that was deduced from the series of partially overlapping sequences obtained for the various peptides isolated from the tryptic (T; second line) and staph protease (S; third line) fingerprints.

COOH-Terminal Aα-Chain Sequence Homology. The first two lines of Figure 8 compare the structures of human and equine Aα chains, respectively, in the region corresponding to the human Aα residues 240–517. The structures have been aligned with gaps introduced to maximize the homology between the two sequences. Reported data for the bovine (Henschen et al., 1980; Chung et al., 1982) and rat (Crabtree et al., 1985) Aα chains, in this same region, are included in the third and fourth lines, respectively, for comparison. The

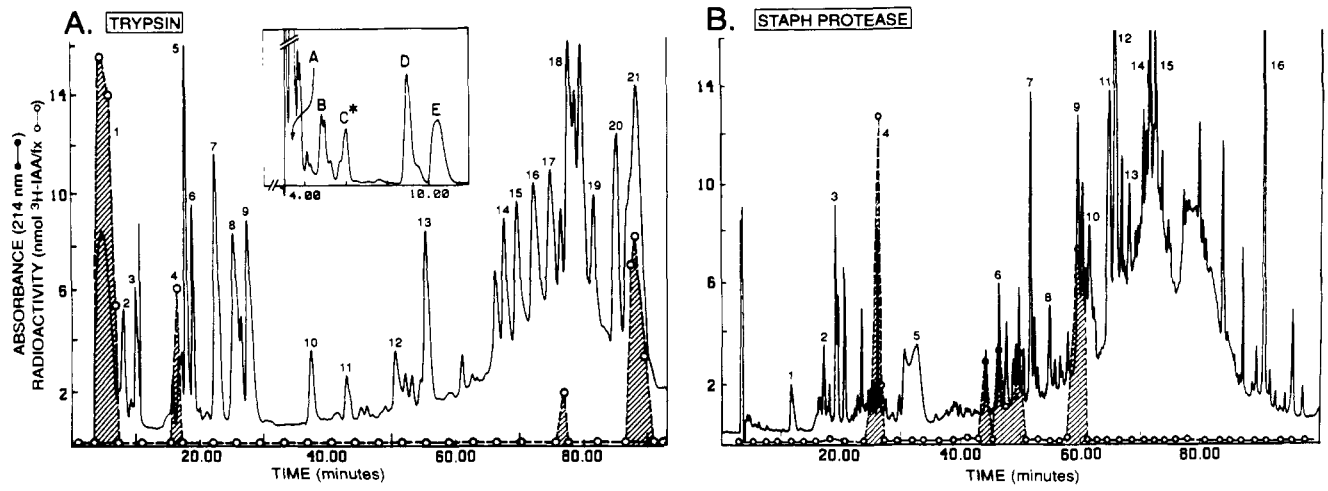

FIGURE 6: Isolation of tryptic and staph protease EqAαCNBr peptides by reversed-phase HPLC. (Panel A) 1.14 mg of reduced and alkylated, trypsin-digested EqAαCNBr (see Figure 5, pool II), containing 93.6 nmol of incorporated ^{3}H-IAA, was dissolved in 1.2 mL of buffer A and subjected to C-4 reversed-phase HPLC as described in the text. One-milliliter fractions were collected. The 214-nm absorbance profile is indicated by the solid line. Absorbance at full scale (AUFS) is 2.0. Peaks 1–21 are numbered consecutively according to their relative retention times. The elution profile of cysteine-containing peptides, determined as ^{3}H-SCMC by counting 4% aliquots of effluent fractions, is indicated by the dashed line, and peaks of radioactivity are hatched. Inset: Approximately 75% of the total material of fraction 6 (see peak 1) was resuspended in 100 μL of H_2O and rechromatographed on a C-18 column as described in the text; 0.5-mL fractions were collected. The 214-nm absorbance profile is shown (AUFS = 0.1), and the peaks obtained are labeled, consecutively, by letter (A–E) according to their relative retention times. The single peak of radioactivity is indicated by an asterisk and was localized by counting 8% aliquots of effluent fractions. (Panel B) 0.93 mg of reduced and alkylated, staph protease digested EqAαCNBr, containing 64.3 nmol of incorporated ^{3}H-IAA, was dissolved in 0.2 mL of buffer A and subjected to C-18 reversed-phase HPLC as described in the text. One-milliliter fractions were collected. The 214-nm absorbance profile is shown (AUFS = 1.0), and the elution of cysteine-containing peptides (determined by counting 10% aliquots of effluent fractions) is illustrated as described for panel A. The partially resolved peptides that eluted with retention times of 60–75 min were rechromatographed (not shown) as described in the text, prior to NH_2-terminal sequencing.

sequence homology between equine and human Aα chains, in the alignment illustrated, is 63% for identical amino acid matches (boxed in Figure 8) and significantly higher if conservative substitutions (i.e., Lys/Arg and Asp/Glu) are considered.

Discussion

The present study describes the partial structural characterization of the Aα chain of equine fibrinogen. The region analyzed is of particular interest because it corresponds to the Aα-chain cross-linking domain of human fibrinogen that includes the glutamine acceptor region, CNBr VIII (Cottrell et al., 1979), and an alleged donor lysine region, CNBr IX (Corcoran et al., 1980). This study was prompted by a concern that the serum used for the growth of hybridoma cell lines secreting monoclonal antibodies to these Aα-chain cross-linking regions might contain cross-reactive animal fibin(ogen) degradation products which would serve to bias the selection of cell lines.

In order to establish whether equine fibrinogen contained a region that might be structurally similar to the human Aα-chain cross-linking domain, we tested for cross-reactivity between a polyclonal antiserum to the acceptor region, CNBr VIII, and the equine Aα chain. Cross-reactivity was observed (Figures 1 and 2; Table I), and this finding of immunologic similarity was employed to isolate the equine counterpart of human CNBr VIII (Figures 3–5).

The equine fragment, designated EqAαCNBr, was similar to its homologous human peptide in that it was extremely hydrophilic, with a high serine and glycine content (data not shown). This allowed for an efficient, two-step purification procedure using hydrophobic chromatography (Figure 4) and reversed-phase HPLC (Figure 5).

Multiple forms of the EqAαCNBr, ranging in size from 32K to 38K, were isolated by HPLC, and all of these cross-reacted with the antiserum to human CNBr VIII (Figure 5, inset). NH_2-terminal sequence analyses of the material in pools I and II (Table II) revealed the same NH_2-terminal structure, so it was inferred that COOH-terminal Aα-chain heterogeneity was responsible for the observed chromatographic differences between the EqAαCNBr fragments. This is consistent with the reported chromatographic heterogeneity of equine fibrinogen (Finlayson & Mosesson, 1964) and with the COOH-terminal Aα-chain heterogeneity that characterizes human fibrinogen preparations, presumbly due to prior proteolytic cleavage (Mosesson et al., 1972).

As shown in Table II, the two predominant EqAαCNBr fragments sequenced poorly, with wide variations in yield observed, particularly at PTH-glycine, proline, and serine residues. Similar difficulties have been reported when sequencing derivatives from the CNBr VIII region of human fibrinogen (Doolittle et al., 1977; Sobel et al., 1983, 1988b; Henschen et al., 1979; Takagi & Doolittle, 1975), and these have been attributed to this region's unusual composition which, as noted above, EqAαCNBr shares.

Following tryptic and staph protease digestion, the peptides released from EqAαCNBr were separated by reversed-phase HPLC (Figure 6), and the resulting fingerprints were characterized by amino acid analysis and NH_2-terminal sequencing (Table III and Figure 7). Staph protease cleavage at four different glycine residues was an unexpected finding (Figure 7), though, interestingly, anomalous cleavage adjacent to a serine residue has been noted during digestion of human CNBr VII by this same enzyme (Strong et al., 1979).

When the sequence of EqAαCNBr, determined from available peptide overlaps (Figure 7), was subjected to computer alignment against the entire 625-residue Aα-chain sequence, the segment of greatest homology was found to correspond to the region represented by CNBr VIII and IX, within the COOH-terminal two-thirds of the human Aα chain, with identity in 169 and 262 possible matches. These data are summarized in the first 2 lines of Figure 8, which presents a slightly different alignment (with identity in 161 of 256 possible matches) that is based on the one reported for the rat

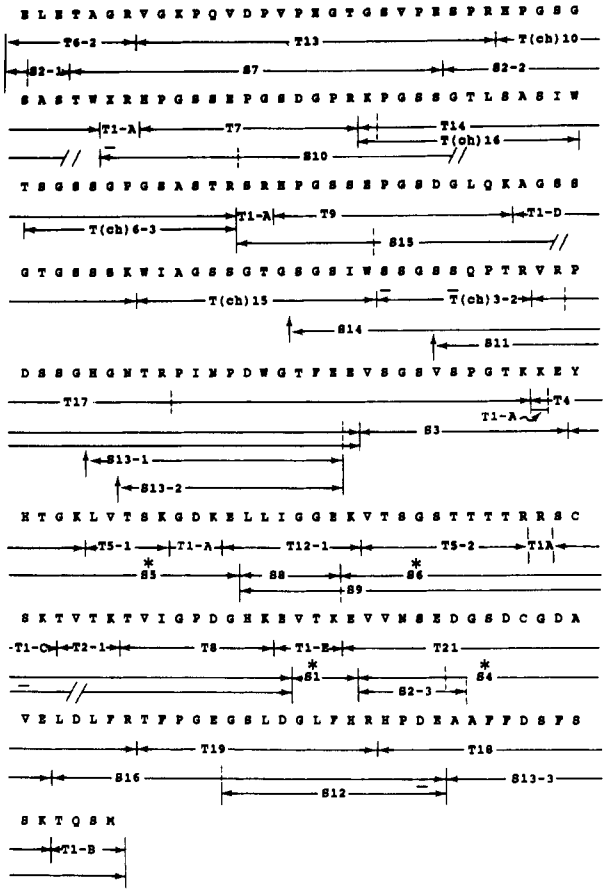

FIGURE 7: Data used to deduce the partial sequence of EqAαCNBr. Tryptic peptides (Figure 6A and Table III) are depicted schematically with their overlapping staph protease peptides (Figure 6B) positioned underneath. Unless otherwise indicated, all the residues bracketed within arrowheads were positively identified by automated Edman degradation. Dashes within a peptide sequence denote residues for which identification was ambiguous. A break within a peptide sequence indicates the last residue before quantitative limitations rendered subsequent data uninterpretable. The complete sequences of peptides S2-2 and S-15 could not be determined due to insufficient material after two trials, and as a result, overlapping sequence data were not obtained for a part of these two regions. Asterisks denote overlaps that were positioned on the basis of compositional data. (In the case of the 36-residue peptide, S-9, the sequence became weak after cycle 25, and residues 26–36 were inferred from the peptide's total composition.) Vertical dashed lines identify sites of incomplete cleavage, while the four vertical arrows point to anomalous cleavages, by staph protease, at glycine residues.

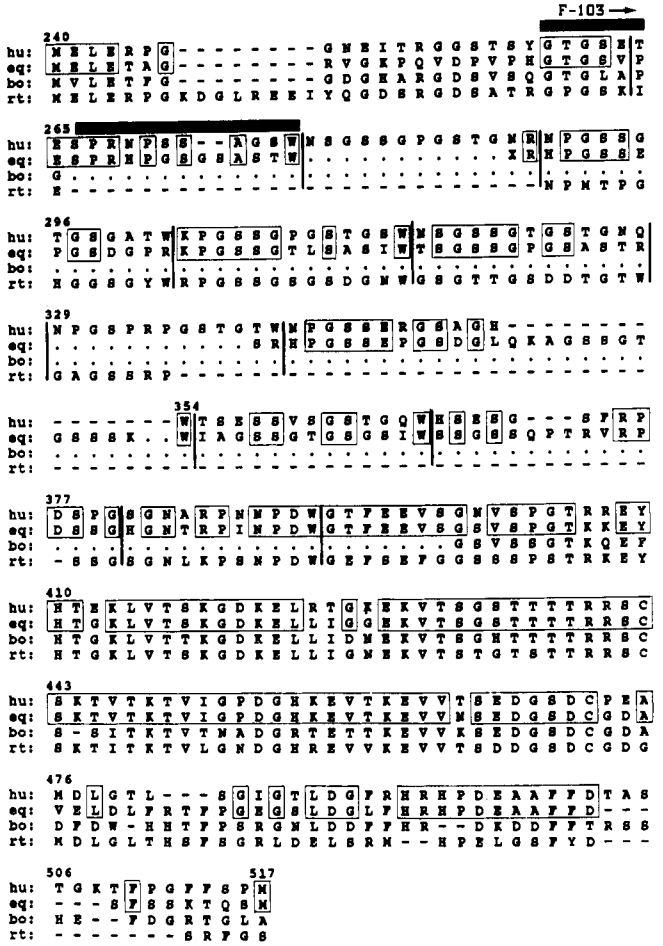

FIGURE 8: Comparative structures of human, equine, bovine, and rat Aα chains in the region Aα 240–517. The partial sequences of human (hu), equine (eq), bovine (bo), and rat (rt) Aα chains are shown aligned on the basis of their structural similarities. The alignment of the four sequences is based on the gaps reported to generate maximal homology between the rat and human Aα-chain sequences (Crabtree et al., 1985). Data for the human, bovine, and rat structures were taken from reported studies (Lottspeich & Henschen, 1978; Henschen et al., 1978, 1979, 1980; Watt et al., 1979; Kant et al., 1983; Chung et al., 1982; Crabtree et al., 1985), and the equine sequence was deduced from the data presented in this report, as summarized in Figures 6 and 7 and Table III. Residues that have not yet been identified (in the bovine and, possibly, equine sequences) are indicated by dots. Gaps, introduced to maximize homology among the various structures, are indicated by dashes, and shared residues, in the human and equine sequences, are boxed. The horizontal black bar identifies the region Aα 259–276 that, in the human sequence, includes the epitope recognized by the monoclonal antibody F-103 (Ehrlich et al., 1983). Vertical lines mark the 10, tryptophan-containing, modified repeats that characterize the mid portion of the human Aα chain (Doolittle et al., 1979).

and human Aα chains by Crabtree and co-workers (Crabtree et al., 1985). In this alignment, three segments of the equine Aα chain (depicted by dots in the equine sequence) are not represented by overlapping peptide data. It may be that some peptides escaped detection either due to poor recovery under the chromatographic conditions employed or because they failed to sequence due to the presence of blocked NH_2-terminal residues. A second possibility is that these "missing" regions are, in fact, represented on peptides that are already accounted for and reflect the presence of inserted, repeating sequences within this portion of the equine Aα chain. This would be consistent with recently reported findings for the structure of lamprey Aα chains (Wang et al., 1989) and would also explain EqAαCNBr's larger size relative to its human counterpart, CNBr IX+X. Although the definitive structure of this portion of the equine Aα chain may have to await elucidation by gene cloning techniques, the data presented here not only support our findings of immunologic cross-reactivity between equine and human Aα chains but also provide strong evidence to indicate that a significant proportion of the COOH-terminal two-thirds of the fibrinogen Aα chain is highly conserved among various animal species.

Several important issues are raised by the comparative data presented in Figure 8. The first applies to the development of hybridoma cell lines that secrete antibodies to Aα-chain cross-linking regions, and in this regard, sequence information for the structure of mouse Aα chains would be extremely useful. These data, however, are not yet available, either from gene cloning or from traditional protein sequencing techniques. In their absence, we have assumed, for discussion purposes, that the structures of mouse and rat Aα chains are similar. To date, there are relatively few reports describing the isolation of murine monoclonal antibodies to human Aα chain cross-linking regions (Ehrlich et al., 1983; Francis et al., 1985;

Thurlow et al., 1987; Hoegee-de Nobel et al., 1988), and for only two of these antibodies have the epitopes been localized to defined sequences; the immunoglobulins F-103 and F-102 recognize determinants in the vicinity of Aα 259–276 and 540–554, respectively (Ehrlich et al., 1983). The data presented in Figure 8 indicate that most of the region that contains the F-103 epitope (highlighted by the black bar) is absent from the structure of rat Aα chains and that, moreover, a significant proportion of this same region is not conserved in the equine molecule. (Its structure in the bovine Aα chain is not yet reported.) Similarly, the F-102 epitope is missing from bovine fibrinogen and is relatively nonconserved in the rat Aα chain (not shown). These findings strongly suggest that the antibodies F-103 and F-102 were isolated only because they could not be absorbed by cross-reactive antigens present in the serum-supplemented culture medium. This raises the possibility that the other reported, but as yet uncharacterized, antibodies (Francis et al., 1985; Thurlow et al., 1987; Hoegee-de Nobel, 1988) may illustrate this same phenomenon and be directed either against the F-103 and F-102 epitopes or against similarly, nonconserved COOH-terminal Aα-chain determinants (Ehrlich et al., 1983). Interestingly, this possibility is strengthened by the recent description of an anti-fibrinogen T-cell hybridoma that recognizes the region Aα 551–578 (Lee et al., 1988), a large portion of which appears to be deleted from the rat Aα chain (Crabtree et al., 1985).

In order to overcome this potential limitation in the repertoire of anti-COOH Aα-chain antibodies, consideration of conserved and nonconserved sequences among the four species shown in Figure 8 might help to better predict which combination of host animal and source of serum supplement would optimize not only the generation but also the isolation of antibodies targeted to specified determinants. Where the potential for cross-reactive interference by fragments in horse or bovine serum exists (e.g., see Aα 342–350 or 492–502), rat serum might be successfully employed as an alternative supplement, or immunologic adsorption methods could be developed to remove fibrin(ogen)lytic fragments from horse serum prior to its addition to the culture medium.

A second issue raised by the data in Figure 8 concerns the functional specificity implied by the presence of conserved sequences within the COOH-terminal portion of the Aα chain. While much of the tryptophan repeat region (Doolittle et al., 1979) appears to be preserved in the equine Aα chain (Figure 8, vertical lines), the two glutamine residues (Aα 328 and 366) that are thought, on the basis of in vitro studies, to impart this region of the human Aα chain with factor $XIII_a$ cross-linking activity (Cottrell et al., 1979) are not strictly conserved. Moreover, while glutamine residues are absent from the few repeats that characterize the rat Aα chain, recent findings for the structure of the lamprey Aα chain indicate the presence of a glutamine residue in each of 23 repeats that appear to be similar to the human prototype (Wang et al., 1989). While these observations raise questions about factor $XIII_a$'s substrate specificity during α-chain cross-linking, it may be that other, as yet unidentified, glutamine residues have functional activity in vivo. The presence of a conserved glutamine residue, in rat, bovine, and human Aα chains, corresponding to Aα 564, is of interest in this regard.

One of the most striking features of the data presented in Figure 8 is the structural similarity among the four species in the region Aα 388–474 and, particularly, between residues 428 and 464. This segment of the human Aα chain contains an intrachain disulfide bridge (Doolittle et al., 1978), and while the functional significance of this conserved region in mammalian fibrinogens is unclear, the high degree of homology observed suggests both specificity and a role of considerable importance. In view of this, reports implicating COOH-terminal Aα-chain regions in processes related to immunosuppression (Edgington et al, 1985; Plow & Edgington, 1986) and wound healing (Mosher, 1975) may be of interest.

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Peptides Corresponding to the Second Repeated Sequence in MAP-2 Inhibit Binding of Microtubule-Associated Proteins to Microtubules

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ABSTRACT: Bovine brain high molecular weight microtubule-associated proteins (MAPs) can be displaced from assembled tubules by peptides corresponding to the second of three nonidentical repeated sequences in mouse MAP-2. The octadecapeptide m_2 (VTSKCGSLKNIRHRPGGG) can release MAP-1b from MAP-containing microtubules, and the extended second-sequence peptide m_2' (VTSKCGSLKNIRHRPGGGRVK) displaces MAP-1a and MAP-1b as well as MAP-2a and MAP-2b. Peptides m_2 and m_2' stimulate tubulin polymerization in the absence of MAPs or microtubule-stabilizing agents, and m_2' acts as a competitive inhibitor of radiolabeled MAP-2 binding. The dissociation constant for MAP-2 binding to taxol-stabilized tubules was 3.4 μM in the absence of m_2' and 14 μM in the presence of 1.5 mM of the m_2' peptide. We estimate that the inhibition constant for peptide m_2' is about 0.5 mM, about 100 times lower than for the K_m of MAP-2. These observations suggest that the second repeated sequence in MAP-2 may represent an important recognition site for MAP binding to microtubules and that other structural features within MAP-2 may reinforce the strength of MAP-microtubule interactions.

Microtubule-associated proteins (MAPs) exhibit one of several properties: the ability to copolymerize with tubulin during microtubule assembly, the capacity to utilize tubulin or another MAP as substrates for enzyme-catalyzed modification, or the use of microtubules as the architectural framework for motility (Olmsted, 1986; Purich & Kristofferson, 1984). The first property is shared by the high molecular weight proteins (MAP-1 and MAP-2) as well as the τ proteins, and these proteins remain associated with reassembled microtubules during the course of microtubule protein purification. Recently, the cDNA-derived amino acid sequences of the murine MAP-2 (Lewis et al., 1988) and the murine τ (Lee et al., 1988) proteins have been defined, and these proteins were both found to contain a related triad of imperfectly repeated octadecapeptide sequences in their tubule binding regions. Oligopeptide analogues of the repeated sequences in murine τ and a 190-kDa bovine adrenal gland MAP can promote microtubule assembly (Ennulat et al., 1989; Aizawa et al., 1989). Likewise, we have demonstrated that analogues of the second repeated sequence of murine MAP-2 can promote microtubule self-assembly (Joly et al., 1989).

While several peptides corresponding to sequences in fibrous MAPs can stimulate microtubule assembly, very little is known about whether these synthetic peptides constitute the entire site necessary for MAP binding to microtubules. If the repeated sequences are indeed the primary sites of interaction, then those promoting tubule assembly in the absence of MAPs may also displace MAPs from microtubules or block their binding to microtubules. Moreover, we were motivated to learn whether a particular peptide and MAP display competitive

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