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Truncated forms of human complement Factor H

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By the use of Western-blot analyses with polyclonal anti-(Factor H) antibodies, two low-M_r protein species of M_r 41000 and 37000 under non-reducing conditions and 43000 and 40000 under reducing conditions are consistently detected together with the well-known 155000-M_r Factor H in human plasma and serum. These two additional species are also found in plasma, urine and synovial fluids. The 41000-M_r species but not the 37000-M_r species is detected by a monoclonal anti-(Factor H) antibody directed at the *N*-terminal part of Factor H. The 37000-M_r species but not the 41000-M_r species is detected by a monoclonal anti-(Factor H) antibody directed at the *C*-terminal part of Factor H. The 41000-M_r and 37000-M_r species are different from the well-characterized 36000-M_r *N*-terminal tryptic fragment of Factor H. They are likely to represent translational products of the short Factor H mRNA species of 1.8 kb and 1.2–1.5 kb occurring in human liver that we have recently described.

INTRODUCTION

Factor H is a single-chain soluble (Whaley & Ruddy, 1976*a*) and membrane-bound (Malhotra & Sim, 1985; Demares *et al.*, 1987) glycoprotein of M_r 155000, which plays a key role in the regulation of the alternative pathway of the complement system (Whaley & Ruddy, 1976*b*). Factor H controls the extent of proteolytic inactivation of C3b, the major complement activation product, by the serine proteinase Factor I (Pangburn *et al.*, 1977). Recent sequence analyses have shown that Factor H is structurally similar to other functionally similar proteins of the complement system, namely complement receptor type 1, C4-binding protein, decay-accelerating factor and complement receptor type 2. These proteins all contain internal repeat units of approx. 60 amino acid residues having a characteristic framework of highly conserved residues (Reid *et al.*, 1986; Ripoche *et al.*, 1988). The primary structure of Factor H is made up of 20 of these repeat units (Ripoche *et al.*, 1988). The relationship between these proteins is further emphasized by the fact that their structural genes are linked in a gene cluster (designated the RCA cluster) on chromosome 1 (Rodriguez de Cordoba *et al.*, 1985). In Northern-blot analyses of human liver mRNA with Factor H cDNA probes, it has been observed that, in addition to the 4.3 kb mRNA encoding the familiar 155000-M_r form of Factor H, two further mRNA species of 1.8 kb and 1.2–1.5 kb hybridize to Factor H probes (Ripoche *et al.*, 1987; Day *et al.*, 1987; Schwaeble *et al.*, 1987). A single low-M_r form of Factor H protein occurring in plasma has been identified in previous work (Schwaeble *et al.*, 1987).

In the present paper we provide evidence that, in addition to the familiar 155000-M_r Factor H molecule, two truncated forms of this protein occur in blood and other body fluids and may represent translational products of both of the short mRNA species present in human liver.

MATERIALS AND METHODS

Antisera and proteins

Human normal plasma (or serum) samples were obtained from the blood transfusion centre (Bois-Guillaume, France). Synovial fluids and sera from arthritis patients were kindly provided by Professor Deshayes and Professor Leloët (Rheumatology Unit, C.H.R., Bois-Guillaume, France). Polyclonal anti-(Factor H) antibodies were obtained as described previously (Ripoche *et al.*, 1984). Rabbit antibodies against C3, C5 and Factor B were obtained in our laboratory by immunizing rabbits with purified antigens. Anti-(α_1 acid glycoprotein) antibody was generously given by Dr. J. P. Lebreton. Monoclonal anti-(Factor H) antibodies, MRC OX23 and MRC OX24, were obtained as described previously (Sim *et al.*, 1983). These are monoclonal antibodies directed against epitopes present at the *N*-terminal part of Factor H. Monoclonal anti-(Factor H) antibody 3D11.7 is directed against epitopes present at the *C*-terminal part of Factor H (V. Koistinen, unpublished work). The specificity of these monoclonal antibodies was checked by Western-blot analysis of trypsin-digested Factor H (see below). M_r markers were purchased from Bio-Rad Laboratories (via Touzart et Matignon, Paris, France).

Human Factor H was purified and digested with trypsin as described previously (Sim & DiScipio, 1982; Ripoche *et al.*, 1984). Where indicated, body-fluid samples to be analysed by Western blotting were enriched in Factor H-related material by affinity chromatography on polyclonal anti-(Factor H) antibodies coupled to Sepharose 4B (Pharmacia). A 1–10 ml portion of sample was loaded on to 20 ml of resin containing 10 mg of

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis.

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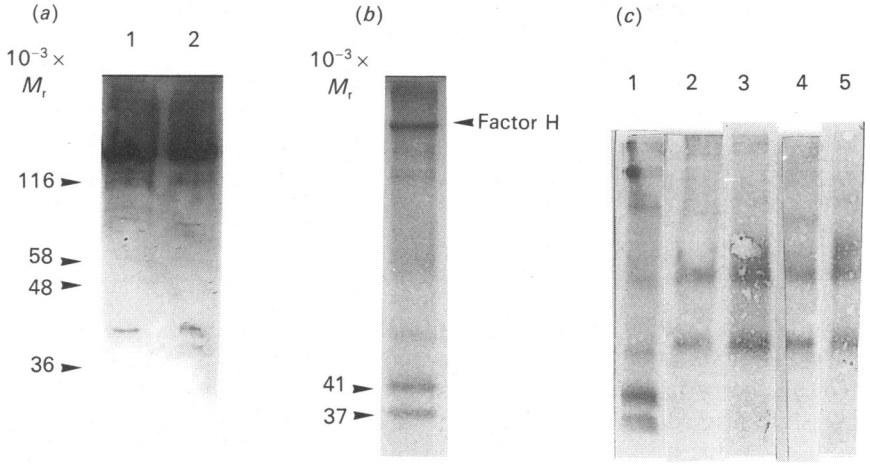

Fig. 1. Detection of two low-M_r forms of Factor H in human serum

(*a*) Normal human serum (2.5 μl) was subjected to SDS/PAGE under non-reducing conditions and then analysed by Western blotting with a polyclonal anti-(Factor H) antibody, as described in the Materials and methods section. Twelve sera were analysed. Ten out of the 12 (track 2) show the two low-M_r species and two (track 1) show only the larger low-M_r species (see the text for details). (*b*) Normal human serum was enriched in Factor H-related material by chromatography on an anti-(Factor H) antibody immuno-affinity column as described in the Materials and methods section. A 50 μg portion of this material was subjected to SDS/PAGE under non-reducing conditions and analysed by Western blotting with a polyclonal anti-(Factor H) antibody as primary antibody. The Figure shown is the result of a typical experiment. Of six normal sera analysed by this method so far, all have shown the two low-M_r species in addition to intact 155000-M_r Factor H molecules. (*c*) The same material as in (*b*) was subjected to SDS/PAGE under non-reducing conditions and analysed by Western blotting with various irrelevant polyclonal antibodies as primary antibodies. Track 1: polyclonal anti-(Factor H) antibody. Track 2: polyclonal anti-C3 antibody. Track 3: polyclonal anti-C5 antibody. Track 4: polyclonal anti-(Factor B) antibody. Track 5: polyclonal anti-(α_1 acid glycoprotein) antibody.

IgG/ml of Sepharose. The column was washed with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.2) containing 20 mM-EDTA and 50 mM-6-aminohexanoic acid until the A_{280} was below 0.01. Bound material was eluted with 1 M-acetic acid in distilled water, dialysed against the washing buffer and concentrated to the starting volume.

Western-blotting experiments

Appropriate samples were subjected to SDS/PAGE, under non-reducing or reducing conditions, according to the procedure of Laemmli (1970). Proteins were electrotransferred on to nitrocellulose sheets (Schleicher and Schuell, via CERA Labo, Aubervilliers, France) as described by Towbin *et al.* (1979). Blots were quenched with Blotto [5% (w/v) dried milk and 0.01% (w/v) merthiolate in phosphate-buffered saline] and washed with phosphate-buffered saline containing 0.1% (v/v) Tween 20. All antisera were diluted in Blotto. Blots were developed by immuno-peroxidase staining, with diaminobenzidine as a substrate. Peroxidase-conjugated anti-(rabbit immunoglobulin) and anti-(mouse immunoglobulin) antibodies were purchased from Nordic Laboratories (via Tebu, Le Perray en Yvelines, France). In some experiments gold-conjugated goat anti-(rabbit immunoglobulin) antibodies (Janssen Life Sciences Products, via Tebu) were used as second antibody and processing of the blots was carried out as recommended by the manufacturer.

Northern-blotting experiments

Northern-blot analyses of polyadenylated mRNA from human liver were done essentially as described by Thomas (1980), with two different Factor H-specific cDNA probes: R2a (Day *et al.*, 1987) and B38-1 (Ripoche *et al.*, 1987, 1988).

RESULTS AND DISCUSSION

Multiple samples of human sera from normal individuals were analysed, under non-reducing conditions, by Western-blotting experiments with a polyclonal anti-(Factor H) antibody. By this method, two low-M_r protein species of M_r 37000 and 41000 were detected in addition to the familiar 155000-M_r Factor H (Fig. 1*a*). Of 12 samples from normal individuals, ten showed these two low-M_r species and two only the larger 41000-M_r species.

In order to improve the sensitivity of the detection, samples to be tested were enriched by immuno-adsorption on polyclonal anti-(Factor H) antibodies coupled to Sepharose as described in the Materials and methods section. By using this approach, the two low-M_r protein species were readily and consistently detected in human plasma and serum (Fig. 1*b*). The two individuals showing only the larger 41000-M_r low-M_r species were not tested after this enrichment step, and therefore the possibility that they do only express the 41000-M_r species and not the 37000-M_r one remains to be tested.

Control experiments, in which material immuno-adsorbed on polyclonal anti-(Factor H) antibodies coupled to Sepharose was analysed with irrelevant antibodies, did not show the two low-M_r species, but did show all contaminating additional minor bands (Fig. 1*c*). These results indicate that the two low-M_r protein species, detected with the polyclonal anti-(Factor H) antibodies, are antigenically related to Factor H.

In reducing conditions the electrophoretic mobilities

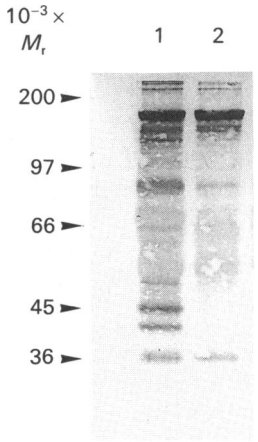

Fig. 2. Evidence that the two low-M_r forms are different from the 36000-M_r tryptic fragment of Factor H

Track 1: 50 μg of the same material as in Fig. 1(*b*) was subjected to SDS/PAGE under reducing conditions and analysed by Western blotting with a polyclonal anti-(Factor H) antibody as primary antibody. Track 2: 25 μg of trypsin-digested Factor H [2 min at 37 °C, 1% (w/w) trypsin, reaction stopped with a 2-fold molar excess of soya-bean trypsin inhibitor over trypsin] was analysed as in track 1.

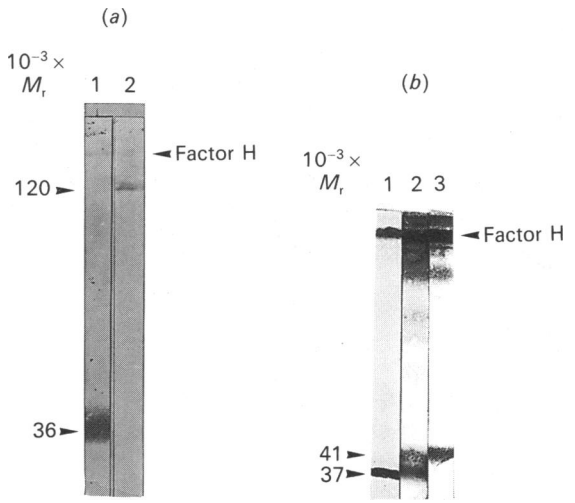

Fig. 3. Characterization of the two low-M_r forms of Factor H with monoclonal anti-(Factor H) antibodies

(*a*) Specificity of 3D11.7 and OX24 monoclonal antibodies was tested by Western-blot analysis of trypsin-digested purified Factor H. A 25 μg portion of trypsin-digested Factor H [2 min at 37 °C, 1% (w/w) trypsin, reaction stopped with a 2-fold molar excess of soya-bean trypsin inhibitor] was subjected to SDS/PAGE under reducing conditions and analysed by Western blot. Track 1: monoclonal OX24. Track 2: monoclonal 3D11.7. (*b*) Normal human serum was enriched in Factor H-related material by chromatography on an anti-(Factor H) antibody immuno-affinity column, as described in the Materials and methods section. A 50 μg portion of this material was subjected to SDS/PAGE under non-reducing conditions and analysed by Western blotting as described in the Materials and methods section with the indicated anti-(Factor H) antibodies as primary antibody. Track 1: monoclonal antibody 3D11.7 (directed to the *C*-terminal part of Factor H). Track 2: polyclonal anti-(Factor H) antibody. Track 3: monoclonal antibody OX24 (directed to the *N*-terminal part of Factor H).

of the low-M_r doublet decreased to apparent M_r values of 43000 and 40000 (Fig. 2). Mild trypsin or plasmin digestion of Factor H gives a fragmentation pattern consisting of two disulphide-linked polypeptides of M_r 120000 (*C*-terminal) and M_r 36000 (*N*-terminal), which are readily detectable when analysed by SDS/PAGE under reducing conditions (Sim & DiScipio, 1982). This type of fragmentation occurs in plasma or in Factor H preparations stored without proteinase inhibitors. Two lines of evidence ruled out the possibility that the 41000-M_r and/or the 37000-M_r species could be related to the 36000-M_r tryptic fragment of Factor H. First, they are detected under non-reducing conditions (Fig. 1). Under non-reducing conditions the 36000-M_r tryptic fragment remains disulphide-bonded to the remaining part of the Factor H molecule (M_r 120000) and is not detected as a separate species (Sim & DiScipio, 1982). Secondly, under reducing conditions (Fig. 2) the 43000-M_r and the 40000-M_r species are clearly distinct from the 36000-M_r tryptic fragment of Factor H. The serum sample used in this experiment was stored without proteinase inhibitors, and in these conditions the proteolytic 36000-M_r fragment analogous to that obtained on tryptic cleavage of Factor H can be observed. The extra 80000-M_r band was not identified but probably corresponds to a further degradation of the 120000-M_r species (Sim & DiScipio, 1982). However clear-cut are these results, the possibility that these low-M_r species are the products of a hitherto unreported degradation pattern of Factor H cannot be definitively ruled out.

These two low-M_r species could be detected in normal urines. They were also detected in sera and synovial fluids from patients with rheumatoid arthritis (results not shown). No relative variations in the ratio of low-M_r species to intact Factor H could be observed by using the Western-blot technique described in the Materials and methods section. More work is required to quantify more accurately the concentrations of these low-M_r species in various body fluids from normal individuals and patients with inflammatory diseases.

Western-blotting experiments, with monoclonal anti-(Factor H) antibodies as primary antibodies, have been decisive in characterizing these two low-M_r species. With monoclonal antibodies (OX23 and OX24) directed at epitopes present at the *N*-terminal part of Factor H (Sim *et al.*, 1983) only the larger low-M_r species of M_r 41000 could be detected (Fig. 3*b*). With a monoclonal antibody (3D11.7) directed at epitopes present at the *C*-terminal part of Factor H only the smaller low-M_r species of M_r 37000 could be detected (Fig. 3*b*). Specificities of monoclonal antibodies OX23, OX24 and 3D11.7 were checked by Western-blotting experiments with trypsin-digested Factor H (Fig. 3*a*). Monoclonal antibodies OX23 and OX24, as expected, recognized the 36000-M_r *N*-terminal tryptic fragment of Factor H, whereas monoclonal antibody 3D11.7 recognized the 120000-M_r *C*-terminal tryptic fragment of Factor H. Taken together, these results show that the 41000-M_r low-M_r species corresponds to a truncated form of Factor H bearing *N*-terminal epitopes, whereas the 37000-M_r low-M_r species corresponds to a truncated form of Factor H bearing *C*-terminal epitopes.

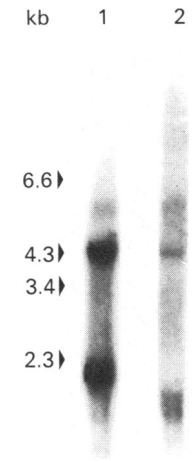

Fig. 4. Northern-blot analysis: detection of two short Factor H-specific messages

Polyadenylated mRNA (5 μg) from human liver was fractionated on a 1% agarose/formaldehyde denaturing gel and analysed by Northern blot with a probe derived from the 5′-end of the Factor H-coding sequence B38-1 (track 1) and a probe derived from 3′-end of the Factor H-coding sequence R2a (track 2).

Sequence analysis of Factor H-specific cDNA clones (Day *et al.*, 1987; Ripoche *et al.*, 1988) and Northern-blot analyses of mRNA from human liver have shown the presence of a short mRNA of 1.8 kb (Ripoche *et al.*, 1987; Schwaeble *et al.*, 1987), containing the coding sequence for an 18-residue leader peptide followed by the first 427 amino acid residues of Factor H and then by an unique Ser-Phe-Thr-Leu sequence (Day *et al.*, 1987; Ripoche *et al.*, 1988). This short message has the same abundance in human liver as the 4.3 kb mRNA coding for the familiar 155000-M_r Factor H. The 1.8 kb mRNA species hybridizes only to a probe B38-1 derived from the 5′-end of Factor H cDNA (Fig. 4, track 1). If translated, the 1.8 kb message would produce a truncated form of Factor H that would have M_r 49100 (without oligosaccharide). This putative product would possess the sequence responsible for the C3b-binding activity and the cofactor activity, since Alsenz *et al.* (1985) have shown that the 36000-M_r *N*-terminal tryptic fragment of Factor H possesses both these activities. This truncated protein would also contain the Arg-Gly-Asp sequence, which is present in the fourth repeat unit of Factor H (Ripoche *et al.*, 1988). The 41000-M_r species may correspond to the translation product of the 1.8 kb message on the basis of its M_r and because it is recognized by monoclonal anti-(Factor H) antibodies, which have all been shown to react with the 36000-M_r *N*-terminal fragment. The discrepancy in M_r between the 41000-M_r protein observed and the 49100-M_r product expected may simply be a feature of the anomalous migration of Factor H on SDS/PAGE. Non-reduced Factor H, of M_r 155000, migrates on SDS/PAGE with an M_r of 120000–130000, and reduced Factor H has an M_r of 160000-175000. Alternatively, the 41000-M_r product may represent a processed (proteolysed) form of the 49100-M_r product. Extensive protein sequencing, particularly at the *C*-terminal end of the 41000-M_r species, will be required to distinguish between these possibilities. The 41000-M_r fragment has also been observed in plasma by Schwaeble *et al.* (1987) and its origin interpreted similarly.

In Northern blotting with Factor H cDNA probes a further mRNA of 1.2–1.5 kb is observed (Fig. 4). This mRNA species is recognized by the 3′-end probe R2a (containing the 3′-terminal half of the Factor H coding sequence), but not by B38-1, which contains the 5′-terminal third of the Factor H coding sequence. No cDNA known to correspond to the 1.2–1.5 kb mRNA has yet been sequenced, and so the precise relationship to the Factor H coding sequence is unknown. The mRNA is likely, however, if translated, to give a product related to the *C*-terminal half of Factor H. The 37000-M_r species seen in Western blotting is recognized by antibodies specific for this region. The coding sequence for a 37000-M_r protein is approx. 1100 bp or less, and so this species is a suitable candidate for the translation product of the 1.2–1.5 kb mRNA.

Protein sequence studies will be required to confirm the origin of these two Factor H-related proteins. Initial attempts at isolation indicate that both species are present in plasma at very low concentration, less than 1/200th of the concentration of full-length (155000-M_r) Factor H.

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