THE Cμ3-DOMAIN OF IgM: ISOLATION AND IDENTIFICATION OF THE INTACT FRAGMENT

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Received May 10,1977

Summary Intact Cμ3-domain was produced by tryptic digestion of purified Fcμ and was isolated by molecular exclusion chromatography under non-dissociating conditions. Purified Cμ3-domain was identified on the grounds of its antigenic reactivity, amino acid sequence and composition and molecular weight. It has a molecular weight of 17,900 and probably comprises the amino acid sequence Gly-326 to Arg-451 of the μ-chain.

Introduction

The hypothesis of Edelman et al. (1) which requires each domain of the immunoglobulins to have one or more effector or structural functions, has been experimentally verified in a number of instances (Table V ref. 2). In order to clearly distinguish these functions it is necessary to either isolate individual domains in pure form or to use immunoglobulins in which specific domains are deleted. We have recently described the isolation and identification of the Cμ4-domain of IgM (3) and have shown it to be directly involved in the cytophylic adherence reaction between E_{ox} IgM$_{rabbit}$ and human lymphocytes (4). This domain has also been shown to carry a Cl^--fixation site (5). We now wish to report on the production, isolation and identification of the intact Cμ3-domain of a monoclonal human IgM.

Materials and Methods

The isolation of the monoclonal IgM and the production and isolation of its $Fc_5\mu$ and Fcμ-fragments have been described in detail (3). Limited tryptic digestion of Fcμ was done according to Hester et al. (6). Purified Fcμ (5 mg/ml in 0.05 M Tris-0.5 M NaCl buffer, pH 8.0) and trypsin (DCC-treated) dissolved in one tenth volume that of Fcμ of 0.001N HCl-0.1M $CaCl_2$ were incubated separately at 37°C (waterbath). After reaching this temperature digestion was started by rapid admixture of enzyme and substrate solutions (E:S ratio of 1:100) and was terminated after 9 minutes by addition of a 10% excess of soybean trypsin inhibitor. The digestion mixture was concentrated to approximately 20 mg/ml (Amicon UM2 membrane) before chromatography on Sephadex G-100 and G-75.

ISSN 0006-291X

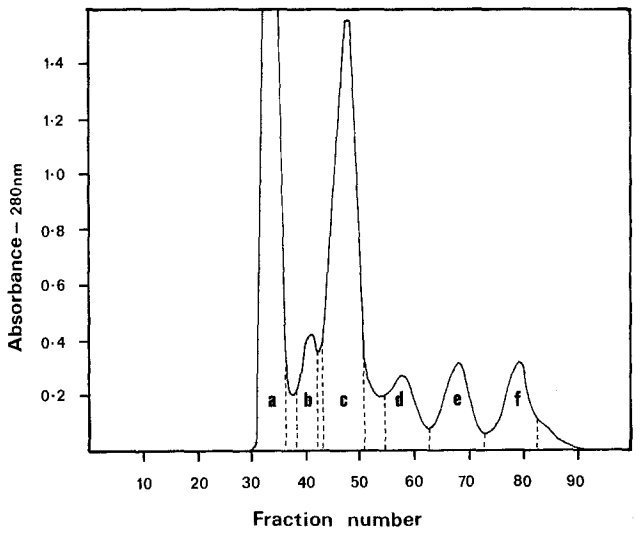

Fig. 1. Elution pattern obtained after chromatography of a tryptic digest (see materials and methods) of 200 mg Fcμ on Sephadex G-75 (column, 2.5 x 90 cm; buffer, 0.05 M Tris-0.5 M NaCl, pH 8.0; flowrate, 25 ml/hour; 5 ml fractions).

Analytical ultracentrifugation, amino acid composition and sequence analyses and sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) of Fcμ fractions were done according to standard methods detailed before (3). A partial specific volume of 0.695 was calculated (12) from the amino acid sequence (11) and carbohydrate (9) compositions of the Cμ3 polypeptide Gly-326 to Arg-451. Ouchterlony double diffusion experiments were carried out in 1% (w/v) I.D. Agar (Oxoid) dissolved in 0.05 M Veronal buffer, pH 8.6 containing 4% (w/v) PEG (mol wt 4000) to enhance the precipitation of low molecular weight antigen-antibody complexes. Monospecific antiserum to the Fcμ-fragment was prepared in rabbits as previously described (7).

Results

Isolation of Cμ3-domain

SDS-PAGE analysis of Fcμ digested for various time intervals indicated that tryptic cleavage for 9 min (37°C) afforded optimum consumption of substrate and production of discreet fragments of lower molecular weight. Gel filtration under non-dissociating conditions of a typical tryptic digest yielded the chromatogram shown in Fig. 1. SDS-PAGE analysis (Fig. 2) of fractions a to d (Fig. 1) showed the first fraction to consist mainly of aggregated low mol wt material migrating close behind the tracking dye (Fig.2c).

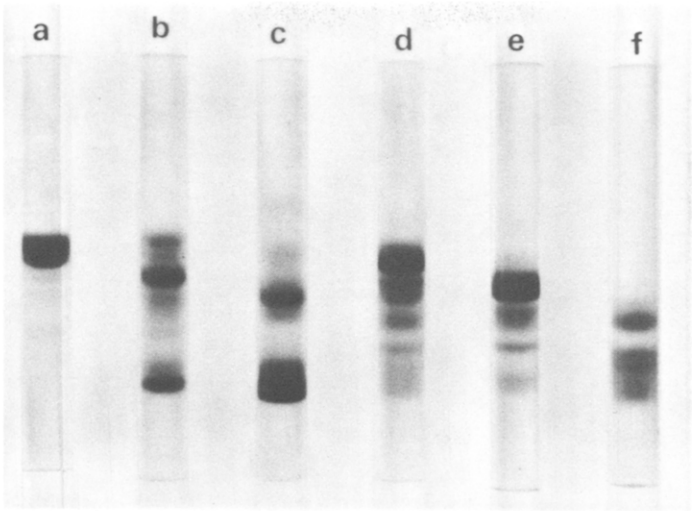

Fig. 2. SDS-PAGE (6% gel) analysis of (a) precursor Fcμ-fragment; (b) tryptic digest (see materials and methods) of Fcμ-fragment; (c) fraction a (Fig. 1); (d) fraction b (Fig. 1); (e) fraction c (Fig. 1); (f) fraction d (Fig. 1). Anode is at the bottom.

Fraction b apparently consists of residual uncleaved Fcμ (Fig. 2d) and the size of the peak confirmed that an optimum period of digestion had been selected. Material in fraction c had an apparent molecular weight of the same order as that of L-chain on SDS-PAGE (Fig. 2e). Because of the anomalously high apparent molecular weight of glycoproteins in SDS-PAGE (8) and the knowledge that Cμ3-domain contains approximately 25% carbohydrate (9), this fraction was examined further. Repeated rechromatography of fraction c protein yielded material (called Cμ3(a)) which was considered of sufficient purity (Fig. 3b) to warrant characterisation. The polypeptide shown in Fig. 3c was a persistent contaminant which was apparently continuously generated during separation procedures. The likely Cμ3 origin of this contaminant (called Cμ3(b)) was indicated by its amino acid composition (Table I) and it is thought to be produced from Cμ3(a) by tightly bound, uninhibited trypsin. Extensive reduction and alkylation (6M guanidine hydrochloride) of Cμ3(a) did not cause the appearance of

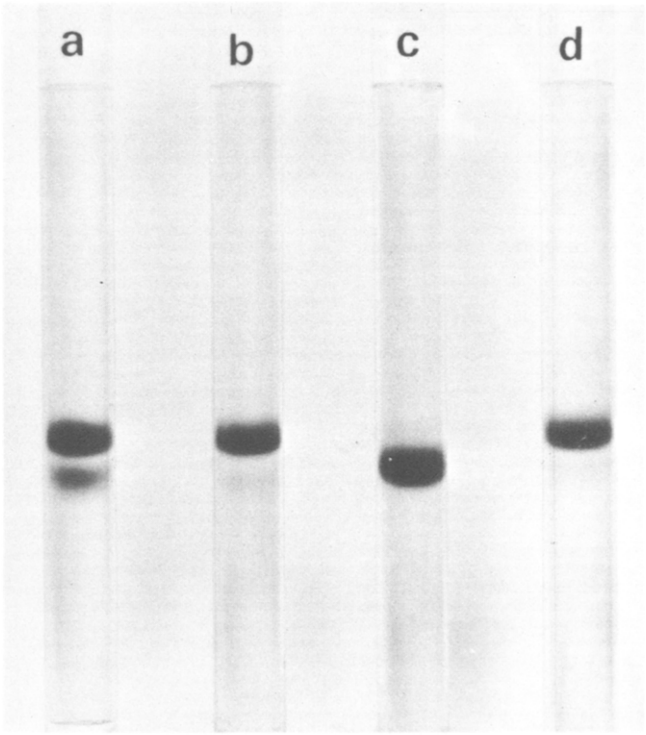

Fig. 3. SDS-PAGE (6% gel) analysis of (a) fraction c (Fig. 1) after first cycle of rechromatography; (b) purified Cμ3(a); (c) purified Cμ3(b); (d) extensively reduced and alkylated Cμ3(a). Anode is at the bottom.

smaller fragments (Fig. 3d) proving that it consists of a single polypeptide chain.

Identification of Cμ3-domain

(a) Amino acid composition

Comparison of the theoretical amino acid composition of the Cμ3 region Gly-326 to Arg-451 (11) with that experimentally determined for Cμ3(a) (Table I) strongly suggested that this fragment was intact Cμ3-domain. Table I also compares the amino acid composition of Cμ3(b) with that of the smaller Cμ3 polypeptide Val-347 to Arg-451 (11). Although a less favourable correlation was obtained in this instance, the absence of methionine residues may perhaps be considered diagnostic.

Table I

Amino acid compositions of Cμ3(a) and Cμ3(b) fragments

	Theoretical[a]	Exp.[b.c.]	Theoretical[d]	Exp.[e.c.]
	Gly-326 to Arg-451	Cμ3(a)	Val-347 to Arg-451	Cμ3(b)
S-CMC	-	2 (2.3)	-	2 (2.0)
Asp	12	12 (12.3)	9	10 (9.9)
Thr	18	18 (18.0)	16	15 (15.0)
Ser	14	14 (13.7)	12	11 (10.7)
Glu	10	11 (10.7)	7	8 (7.7)
Pro	7	7 (7.3)	6	8 (7.7)
Gly	5	5 (4.7)	4	4 (3.8)
Ala	9	9 (8.7)	7	7 -
Val	8	8 (8.0)	7	7 (7.2)
Met	1	1 (0.9)	0	0 (0.1)
Ile	7	7 (7.3)	6	6 (6.1)
Leu	8	8 -	7	6 (6.1)
Tyr	1	7 (6.8)[f]	1	5 (4.5)[f]
Phe	6	6 (6.3)	5	4 (4.4)
Lys	5	5 (5.0)	5	4 (4.1)
His	4	4 (3.7)	4	3 (3.0)
Arg	5	4 (4.4)	4	4 (4.1)

a. Calculated from the μ-chain sequence Gly-326 to Arg-451 of IgM (Ou) (11).
b. Residues per 8 residues of leucine.
c. Experimental values (in brackets) are the means of duplicate determinations and are rounded off to the nearest integer.
d. Calculated from the μ-chain sequence Val-347 to Arg 451 of IgM(Ou) (11).
e. Residues per 7 residues of alanine.
f. The high value obtained for Tyrosine presumably reflects the co-elution of amino-sugars.

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                326              330
IgM(Ou)   -Gly-Leu-Thr-Phe-Gln-Gln-Asn-Ala-Ser-Ser-Met-Cys-

Cμ3(a)     Gly-Leu-Thr-Phe-Gln-Gln-Asn-Ala-Ser-Ser-Met-
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Fig. 4. Partial amino acid sequence of Cμ3(a) and of the μ-chain of IgM(Ou) starting at Gly-326 (11).

(b) Molecular weight determinations

A molecular weight of 17870 (± 630; mean of 12 determinations at three different concentrations and two different rotor speeds) was determined for Cμ3(a). This compares well with the theoretical molecular weight of 18247 calculated from the carbohydrate composition (9) and the amino acid sequence of the Cμ3 polypeptide Gly-326 to Arg-451 of IgM(Ou) μ-chain (11).

(c) Amino acid sequence determination

The amino acid sequence of the first eleven residues of Cμ3(a) was determined by automated Edman degradation and is shown in Fig. 4, together with that of the relevant sequence of the μ-chain of IgM(Ou) (11). This experiment firmly established that Cμ3(a) originated from the amino-terminal half (i.e. Cμ3 area) of Fcμ.

(d) Immunodiffusion experiments

Unambiguous proof of the identity of Cμ3(a) was obtained from its antigenic reaction with a monospecific anti-Fcμ antiserum (Fig. 5). This experiment not only showed the partial antigenic identity of Cμ3(a) with its precursor, Fcμ, but also illustrated its complete antigenic non-identity with purified Cμ4-domain. The identity of Cμ3(b) was confirmed in a similar experiment (not shown).

Discussion

Hester et al. (6) have shown that limited trypsinolysis of monomeric Fcμ will lead to cleavage in the inter-domain area of the molecule. Comparison by SDS-PAGE of our (Fig. 2b) and their digestion products of

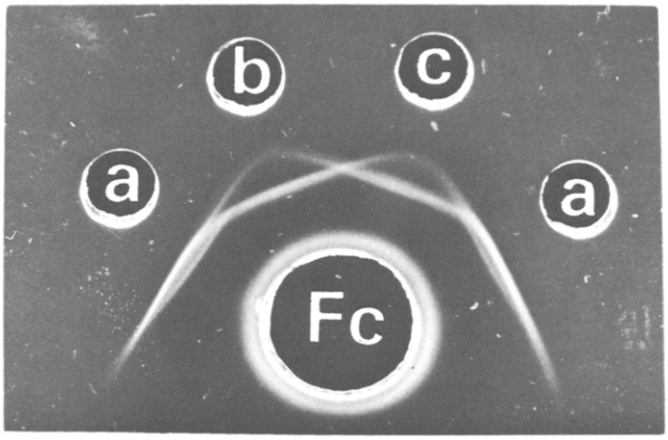

Fig. 5. Double diffusion analysis of (a) Fcμ-fragment; (b) cμ3(a)-domain; (c) cμ4-domain. The large central well contained monospecific rabbit antiserum against Fcμ-fragment.

Fcμ show much the same patterns. In our experience, however, chromatography of this material in non-dissociating buffer presented no problems and led to fairly good separation of the different fragments, the only aggregated material appearing in the first peak (Fig. 1a). The nature and origin of this low molecular weight fraction (Fig. 2c) was not determined in this study but presumably represents the fragmented $C_\mu 4$-domain isolated and characterised before (6). The identity of the μ-chain fragment eluting in fraction c (Fig. 1) was established as Cμ3-domain chiefly by its antigenic reactivity (Fig. 5) and partial amino acid sequence (Fig. 4). Evidence supporting the above identification was also obtained by amino acid composition (Table I) and molecular weight analyses.

We have now isolated both the Cμ3 and Cμ4-domains of IgM in intact form. So far, the effector function of Cμ3-domain remains obscure seeing as both complement fixation (5) and cytophylic activity (4) is apparently mediated only by the Cμ4-domain. Molecular weights of 17,900 and 13,900 have been determined under non-dissociating conditions for the Cμ3 and Cμ4-domains respectively. Moreover, molecular exclusion chromatography

of a synthetic mixture of the two domains show that they do not bind non-covalently in Tris-NaCl buffer. These findings support the conclusion of Hester et al. (6) that non-covalent interaction between the μ-chains of reduced and alkylated IgM is probably mediated by the Cμ2-domain.

Acknowledgments

The authors wish to thank Drs Botes and Visser, N.C.R.L., Council for Scientific and Industrial Research, Pretoria, for amino acid sequence and composition analyses. This work was supported by a grant from the South African Medical Research Council.

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