

A 34-AMINO ACID PEPTIDE OF THE THIRD COMPONENT OF COMPLEMENT MEDIATES PROPERDIN BINDING¹

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In this study, a peptide of 34 amino acids from the M_r 40,000 C terminus α -chain fragment of C3 was found to mediate properdin (P) binding. Treatment of the M_r 40,000 fragment with CNBr generated one major M_r 17,000 fragment that was capable of binding P. Amino acid sequence data placed the M_r 17,000 fragment within residues 1385 to 1540 of the C3 sequence. After analyzing this sequence for highly conserved segments within the C3 from other species (which bind P) and segments of low similarity within human C4, mouse C5, and α_2 -macroglobulin (which do not bind P), a 34-amino acid (1402 to 1435) peptide was synthesized. This synthetic peptide bound to P and inhibited its binding to C3b. In addition, it exhibited negative regulatory activity on the alternative pathway as it inhibited the lysis of rabbit erythrocytes by normal human serum. These results show that the P-binding site is located within the residues 1402 to 1435 of the C3 sequence.

The third component of C, C3, is the most abundant C protein in serum (1.2 mg/ml) and plays a pivotal role in the C system by interacting with other C components and C R. Among the ligands that interact with the different C3 degradation products are: C5; P;³ factors I, H, and B; CR1 (C3b-R); CR2 (C3d-R); CR3 (iC3b-R); CR4, C3a-R; and gp45-70 (for review, see References 1 to 6). In addition to the above ligands, several other proteins such as conglutinins (7, 8) glycoprotein C of herpes simplex virus (9), and laminin (10) have been found to react with different fragments of C3. The nature of these interactions and the involvement of the different binding sites in multiple interactions is largely unknown. P is a molecule of polydisperse nature comprised of a set of cyclic polymers which are constructed from single, highly asymmetric protomers (11, 12). It is one of the six glycoproteins (concentration in serum 5 μ g/ml) comprising the alternative pathway of C, where its principal function is to stabilize the labile C3/C5 convertase, C3bn, Bb. Studies on patients with P deficiency and susceptibility to meningococcal infection have indicated that P is essential for optimal C activation (13, 14). In addition to its enhancement effect on the convertase activity, P pro-

tects C3b against inactivation by factors I and H (15, 16). The exact mechanism by which P stabilizes the C3 convertase and the structural requirements involved in this stabilization are unknown. Toward this, we have made some progress by localizing the P-binding domain in the C3 molecule. Using isolated polypeptides of C3, it was found that the P-binding site is localized within the M_r 40,000 C-terminal α -chain fragment of C3 (17).

In this study, using CNBr fragments of the M_r 40,000 fragment and synthetic peptides, we were able to localize the P-binding site in a segment spanning amino acids 1402 to 1435 of the C3 sequence. A synthetic peptide corresponding to this region, in addition to binding P, exhibited negative regulatory activity in the activation of the alternative pathway.

MATERIALS AND METHODS

Preparation of CNBr fragments of M_r 40,000 fragment. The M_r 40,000 C-terminal α -chain fragment of C3 was purified as previously described (18). The generation and isolation of its CNBr fragments were performed by methods described previously (19).

Peptide synthesis. Peptides representing residues 1402 to 1435 (M34), 1187 to 1205 (P28), and 924 to 966 (K42) (see Fig. 4) of the C3 sequence (20) were synthesized, using an Applied Biosystems 430A synthesizer, employing the standard solid phase method on 4-methylbenzylhydramine resin (21, 22). The peptides were deprotected and removed from the resin by anhydrous hydrogen fluoride in the presence of anisole (23). Next, the peptides were purified by HPLC on a C18 reversed phase column (Vydac, Hesperia, CA) and their amino acid sequence was confirmed by Edman degradation (24). ¹²⁵I labeling of peptide was done by the chloramine T method (25).

Amino acid sequence analysis. Sequence determination was performed in an Applied Biosystems 470A gas phase sequencer with an on-line 120A PTH-analyzer (26).

Assays for binding of P to C3b and synthetic peptides. Three different assays were employed: 1) The direct binding of P to C3b and other C3 fragments, as well as the inhibitory effect of synthetic peptides on this binding, was tested by an ELISA described elsewhere (17). Briefly, microtiter wells were coated (overnight, 4°C) with 50 μ l of C3b or its fragments (10 μ g/ml). Wells were washed and saturated with PBS containing 1% OVA (30 min, 22°C). Next, 50 μ l of P or serum, serially diluted, were allowed to react with the C3b or its fragments (30 min 22°C). Bound P was detected with a monoclonal anti-P antibody (Cytotech, San Diego, CA) or a polyclonal anti-P antibody (Atlantic Antibodies, Scarborough, ME) and the corresponding peroxidase-conjugated rabbit anti-mouse Ig or rabbit anti-goat Ig. For the inhibition of P binding to C3b by synthetic peptides or fluid phase C3b, 20 ng P was preincubated (15 min, 22°C) with different amounts of peptides (16 to 1000 μ M) or C3b (0.3 to 19 μ M) before addition to C3b-coated plates. 2) The direct binding of peptides to P was tested as previously described for the binding of estradiol to its R (27). Briefly, 100 nM P or an equal amount of factor H in PBS was incubated (48 h, 22°C) with 200 nM ¹²⁵I-labeled M34. The protein-peptide complexes were separated from free peptide by gel filtration on a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column (50 \times 1.8 cm) equilibrated in PBS. 3) The effect of synthetic peptides on alternative pathway activation was measured by quantitating the lysis of RaE by normal human serum (28). The inhibition of RaE lysis by the different synthetic peptides was calculated in relation to that obtained with serum in the absence of any peptide (50% lysis).

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³ Abbreviations used in this paper: C3b, major cleavage fragment of C3; P, properdin; RaE, rabbit erythrocytes.

RESULTS

Localization of the P-binding site to a M_r 17,000 fragment of C3. To localize further the P-binding site, CNBr fragments of the M_r 40,000 C-terminal α -chain fragment were generated. The CNBr fragments were isolated by reversed-phase HPLC (Fig. 1). Analysis of the different HPLC fractions by ELISA for binding to P revealed that only the fractions 25 and 26 bound P. SDS/PAGE analysis showed that these fractions contained a M_r 17,000 fragment (data not shown). Edman degradation of this fragment found the 10 amino-terminal residues to be Thr-Gly-Phe-Ala-Pro-Asp-Thr-Asp-Asp-Leu. Comparing this sequence with the amino acid sequence predicted from the C3 cDNA, and considering the location of the CNBr cleavage sites within the M_r 40,000 fragment, the M_r 17,000 P-binding fragment was placed within amino acid 1387 to 1540 of the C3 sequence.

Localization of the P-binding site to a 34-amino acid sequence by use of synthetic peptides. The sequence of the M_r 17,000 fragment was analyzed for segments of high sequence conservation with mouse C3 (29) and rabbit C3 (30), which bind P, and low sequence conservation with the homologous proteins C4 (31), mouse C5 (32), and α_2 -macroglobulin (33), which do not bind P (Fig. 2). The sequence of this fragment is highly conserved within the C3 from different species, but is poorly conserved in the corresponding segment of human C4, mouse C5, and α_2 -macroglobulin (see Discussion). Most striking were the gaps in the segment 1404 to 1432. Consequently, the segment 1402 to 1435 was synthesized and tested for its ability to inhibit the binding of P to C3b. Figure 3 shows a representative experiment of the inhibition of P binding to C3b by this peptide (M34) or by fluid phase C3b. Fluid phase C3b at a concentration of $2.25 \mu\text{M}$ inhibited the binding of P to microtiter plate-bound C3b by 50%. Inhibition P binding to C3b by the peptide M34 ($120 \mu\text{M}$, 50% inhibition) suggested an affinity of M34 for P 55-fold lower than that of fluid phase C3b. Control synthetic peptides (maximum concentration, 1 mM) of similar length representing other areas of C3 had no effect on this interaction. The sequence of these peptides is shown in Figure 4. The direct binding of M34 to P was tested by incubation of ^{125}I -M34 with P or factor H for 48 h at room temperature. The peptide-protein mixtures were then gel filtered through a G-50 Sephadex column equilibrated in PBS. Figure 5 shows that when the P-M34 complex was analyzed by gel filtration

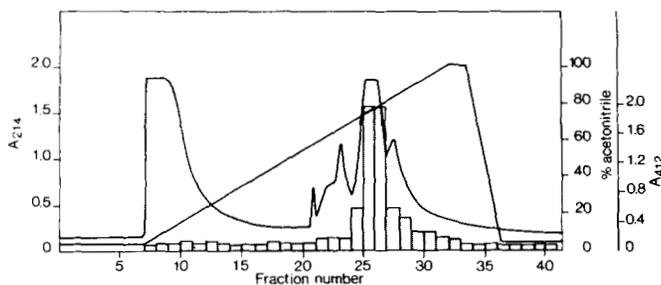


Figure 1. Purification of the CNBr fragments of the M_r 40,000 fragment of C3 and their binding to P. The M_r 40,000 fragment of C3 (3 mg) was incubated with CNBr (30 mg) in 70% formic acid for 24 h at 25°C . After freeze drying, the sample was dissolved in 10% acetic acid and applied to a reversed phase C4 column (Vydac) equilibrated with 5% acetonitrile containing 0.1% trifluoroacetic acid. The CNBr fragments were eluted with a 48-min gradient of 5 to 100% acetonitrile. Fractions of 2 ml were collected, lyophilized, and resuspended in PBS. These fragments were then fixed to ELISA plates and their ability to bind P was performed as described in Materials and Methods.

		1390	1400	1410	1420	1430	1440
HUMAN	C3	TGFAPD	TDDLKQLANGVDRY	ISKYELDKAFSDRNTL	LIYLDKVS	SHKDDCLAFK	VH
MOUSE	C3	*****K**EL**S*****MN*****NK*****E**I**T**E****T****					
RABBIT	C3	***V*****NL**ST*****N*****NK*****I*****REA*****					
HUMAN	C4	S**HALRA**EK*TSLS**V*HF*TE---	GPEV*L-	F*S*PT*RE-	*VG*EAV		
MOUSE	C5	T*IGANEE**RA*VE***QLLTD*QIKDGHVILQL---	NSIPSRDPL*VR*RI*				
α_2 -Macrog		S**I*LKPTV*M*ERSN--HV*RT*VSS-----HVL*****N-QTLS*F*T*L					
		1450	1460	1470	1480	1490	
HUMAN	C3	QYFNVELIQPGAVK	VYAYNLEESC	TRFTRPEKEDGKLNKLCRDELCRCAEENC	FIQ		
MOUSE	C3	*****G*****S*****S*****D**M*S*****HS*****M*					
RABBIT	C3	*****G*****S*****T**Q*****M*S*****HK*****M*					
HUMAN	C4	*EVP*G*V*ASATL*D**P*RR*SV**GAPSKSRL*AT**SA*V*Q**GK*PR*					
MOUSE	C5	*AQLEL*Q*GFLN*ATFT**E*HRPDKQ**MI*SIS--**TR*Q*V*EQA*TV*AD					
α_2 -Macrog		*DVP*RDLK*AI***D**ETD*FAIAE*NAPCSKDLG*APA					
		1500	1510	1520	1530	1540	
HUMAN	C3	KSDDKVTLEERLDKACE	PGVDVYKTRLVQVLS	NDPDE	IM		
MOUSE	C3	Q*QE*IN*NV*****E*TNIK*LD*****T*					
RABBIT	C3	QL*E*I**ND*****L*****K**Q*ERAD*****L*					
HUMAN	C4	RRALERGLQDEGDY*MKF**YYRVEYGFQVK*LRED*RAAFRLF					
MOUSE	C5	QAEV*LAISADS*KE**K*ETA*A**V*ITSATEE*V*VK*TA					

Figure 2. Sequence similarity of the M_r 17,000 fragment of C3 to proteins of the same family. The primary structure of the M_r 17,000 fragment of human C3, predicted from cDNA, has been aligned for maximum sequence similarity with the predicted primary structures of mouse C3 (residues 738 to 892 of the α -chain), rabbit C3 (residues 487 to 520 as predicted from cDNA clone RC3-1), human C4 (residues 1458 to 1622), mouse C5 (residues 1394 to 1543) and α_2 -macroglobulin (residues 1363 to 1451). Where necessary, gaps, as indicated by blank spaces, are introduced to obtain optimal alignment whereas identical amino acids are indicated by an asterisk.

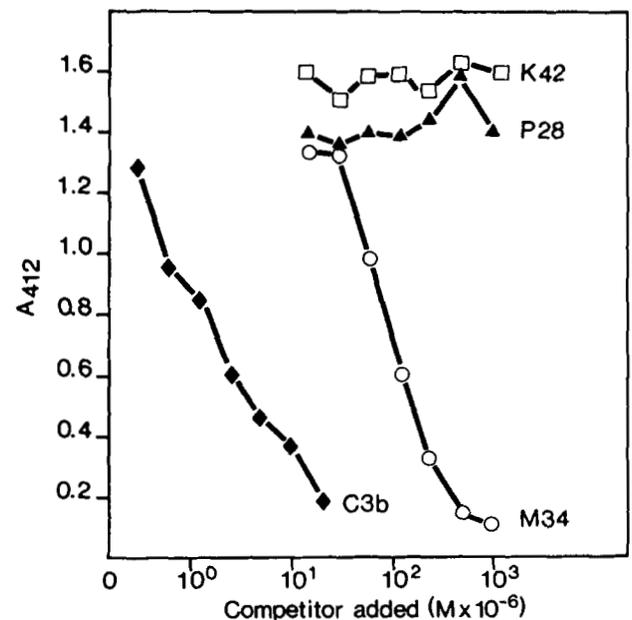


Figure 3. Inhibition of P binding to C3b by synthetic peptides or C3b as determined by ELISA. The various synthetic peptides used were M34 (○), P28 (▲), and K42 (□).

a distinct peak of radioactivity was eluted in the void volume, followed by a large peak that corresponded to the free peptide. In contrast, when gel filtration of the factor H-M34 mixture was performed, the void volume peak was not de-

M34 (1402-1435) GVDRYISKYELDKAFSDRNTLI IYLDSVSHSEDD
 P28 (1197-1224) KFLTTAKDKNRWEDPGLQLYNVEATSYA
 K42 (924-954) TLDPERLGRGEGVQKEDIPPADLSDQVPDTESETRILLQ6TPV

Figure 4. Amino acid sequence of the three synthetic peptides used in this study.

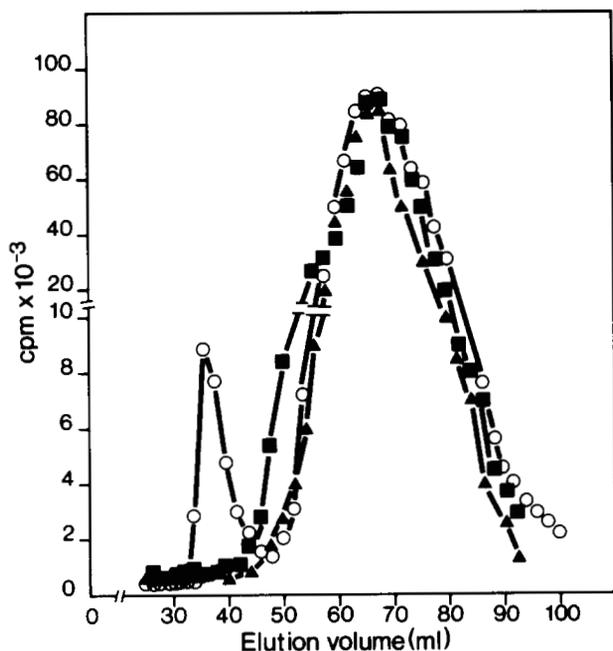


Figure 5. The binding of radiolabeled peptides to P as determined by gel filtration. ^{125}I -labeled M34 (○) or P28 (▲) incubated with P or ^{125}I -M34 (■) with factor H, for 48 h at 25°C and subsequently gel filtered on Sephadex G-50 column. Fractions (2 ml) were collected and counted for radioactivity.

tected. The binding of M34 to P was inhibited by excess cold M34 (data not shown). In addition, when ^{125}I -labeled P28 (19) was incubated with P the elution profile was similar to that of M34-factor H mixture.

Inhibition of the alternative pathway by the M34 synthetic peptide. Because the M34 peptide binds P and inhibits its binding to C3b, we tested the ability of M34 to inhibit the alternative pathway. Figure 6 illustrates the inhibitory effect of the M34 peptide on the lysis of RaE by human serum. A 60-fold molar excess of M34 over the C3 concentration in serum blocked, by 50%, the lysis of RaE via the alternative pathway. The control peptides in the same concentration had no inhibitory effect.

DISCUSSION

There is considerable interest in the multifunctional role of C3 because of its interactions with several serum proteins, cell surface receptors, and proteins from foreign particles. The elucidation of the molecular features related to the different C3 functions requires structural analysis of the C3-ligand interactions. Nine binding sites involving the C3 molecule have been studied: 1) a 6-amino acid C3 peptide (residues 72 to 77) has been found to bind to the C3a-R (34); 2) a 10-amino acid C3 peptide (residues 1227 to 1236) binds to the C3d-R (CR2) (19) and mimics the effect of C3d in the growth of B cells (F. Melchers and J. D. Lambris, unpublished observations); 3) a 21-amino acid C3 peptide (residues 1383 to 1403) binds to the iC3b-R (CR3) (35); 4) a M_r 33,000 C-terminal factor B fragment, as well

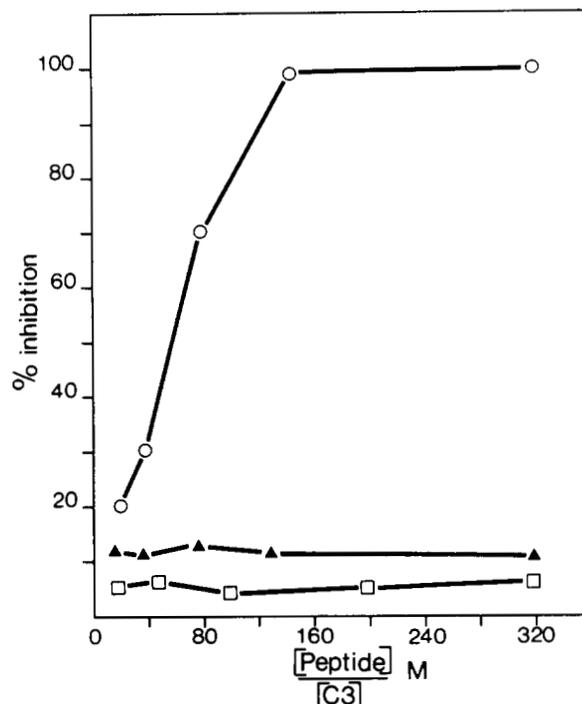


Figure 6. Lysis of RaE by normal human serum in the presence of synthetic peptides. Human serum (20 μl), preincubated with different amounts of M34 (○), P28 (▲), or K42 (□), was incubated with 10^7 RaE for 6 min at 37°C, in 75 μl of gelatin Veronal buffer containing 2 mM Mg^{2+} and 8 mM EGTA. Gelatin Veronal buffer (1 ml), containing 20 mM EDTA, was then added and the hemolytic activity was determined.

as well as the Ba fragment of B, bind C3b (36-38); 5) the conglutinin-binding site in C3 was localized in the carbohydrate moiety located in amino acid 917 (α -chain) of the C3 sequence (39); 6) one of the factor H sites within C3 was localized to a conformational site spanning residues 1187 to 1249 of the C3 sequence (40); and 7) a M_r 30,000 N-terminal factor H fragment binds to C3 and serves as cofactor for its cleavage by factor I (41).

Previous studies of ours have localized the P-binding site in C3 within a M_r 40,000 C-terminal α -chain fragment (17). The aim of this study was to localize further the P-binding site within this M_r 40,000 fragment. After showing that a M_r 17,000 fragment binds P, its primary sequence (residues 1387 to 1540) was compared with the sequences of C3 from other species, which bind P, and to other homologous proteins, such as C4 and α_2 -macroglobulin, which do not bind P. The area-spanning residues 1402 to 1435 of human C3 is highly similar to rabbit and mouse C3, 26/34 (76.5%) and 27/34 (79.5%) common amino acids, respectively. This C3 segment is 6 and 8 amino acids longer than that of C4 and α_2 -macroglobulin, respectively, and shares with them only 10/34 (28%) common amino acids. The mouse C5 segment is 5 amino acids shorter than C3 and has only 4/34 common amino acids with C3. Although the number of sequence identities between P-binding and P-non-binding proteins is not significantly lower in this region, the existence of "gaps" in the P-non-binding proteins was considered very significant and suggests the possible involvement of this region in P binding. This was confirmed by the use of a synthetic peptide covering this region. The peptide M34 bound to P and inhibited its interaction with C3, presumably by competing for binding. The complete inhibition of P binding to C3b by M34 suggests that C3b has only one binding site for P. This is in agreement with

earlier studies on the interaction of P with zymosan-bound C3b in which the stoichiometry was determined to be 1 (42). The appKa of fluid phase C3b for P is 55-fold higher than that of the M34 peptide and 64-fold lower than the Ka of zymosan-bound C3b (42). The affinity differences (if the appKa approximates the Ka) between the zymosan-bound C3b vs fluid phase C3b was also observed in the interaction of factor H with C3b (42, 43). The inhibition of the alternative pathway by this peptide suggests that it may be used to study both the role of P in the alternative pathway during infection and the mechanism of C3 convertase stabilization.

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ERRATUM

In the March 1, 1988 issue of *The Journal*, Volume 140, No. 5, page 1579 in the article by Maria E. Daoudaki, J. David Becherer, and John D. Lambris entitled "A 34-Amino Acid Peptide of the Third Component of Complement Mediates Properdin Binding" mistakes were made in Figure 4 and in the "Materials and Methods" section due to discrepancies in the numbering of amino acid residues of C3. This resulted from the inadvertent inclusion or omission of the signal peptide sequence. The authors have submitted a new figure and legend, which are presented here to clarify this matter.

M34 (1402-1435) GVDRYISKYELDKAFPSDRNTLIYLDKVSHEDD

P28 (1187-1214) KFLTTAKDKNRWEDPGKQLYNVEATSYA

K42 (924-965) TLDPERLREGVQKEDIPPADLSDQVPDTESETRILLQCTPV

Figure 4. Amino acid sequence of the three peptides used in this study. The numbering of the amino acid residues is based on the deduced amino acid sequence of C3 (Ref. 20) after subtracting the signal peptide sequence.

ANNOUNCEMENTS

Immunology Faculty Positions. The Department of Microbiology invites applications for 2 full-time tenure-track positions at any faculty level. Applicants must have a Ph.D. or M.D. and at least 2 years of postdoctoral experience. Successful candidates must establish competitive, funded research programs and help teach medical and graduate students. The Medical College and its Cancer Center are expanding in areas of molecular biology, neurosciences, bone marrow and organ transplantation, gastroenterology, and ophthalmology. Applicants with interests that may complement these areas are encouraged to apply. Send resume, summary of research interests, and names of three references to: Immunology Search Committee, Department of Microbiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. Affirmative action/equal opportunity employer.

Research Associate (M.D. or Ph.D.) to work on the molecular biology of inherited humoral immunodeficiencies in humans. Three years experience in molecular immunology required, especially genomic cloning, DNA and RNA sequencing, tissue culture and immunochemical assays. Position for 2 years beginning immediately. Salary negotiable. Send CV and names of two references to: Personnel Department, Bowman Gray School of Medicine of Wake Forest University, 300 South Hawthorne Road, Winston-Salem, NC 27103.

The Tufts University School of Veterinary Medicine, Department of Comparative Medicine, seeks a molecular biologist with a strong research interest in the immunobiology of resistance to infectious disease. The appointment will be at the Assistant Professor level with a starting date on/around November 1 or by mutual agreement. Responsibilities will include: developing or sustaining an independent research program, participating in an immunology course, and collaborating in an ongoing program concerning the development of transgenic disease-resistant animals. Interested individuals should send curriculum vitae plus the names of three references to:

Dr. Carol L. Reinisch, Chair
Tufts University
School of Veterinary Medicine
Department of Comparative Medicine
136 Harrison Avenue
Boston, MA 02111

Tufts University is an Affirmative Action Equal Opportunity Employer.