Topological mimicry and epitope duplication in the guanylyl cyclase C receptor

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Abstract

Guanylyl cyclase C (GCC) is the receptor for the gastrointestinal hormones, guanylin, and uroguanylin, in addition to the bacterial heat-stable enterotoxins, which are one of the major causes of watery diarrhea the world over. GCC is expressed in intestinal cells, colorectal tumor tissue and tumors originating from metastasis of the colorectal carcinoma. We have earlier generated a monoclonal antibody to human GCC, GCC:B10, which was useful for the immunohistochemical localization of the receptor in the rat intestine (Nandi A et al., 1997, *J Cell Biochem* 66:500–511), and identified its epitope to a 63-amino acid stretch in the intracellular domain of GCC. In view of the potential that this antibody has for the identification of colorectal tumors, we have characterized the epitope for GCC:B10 in this study. Overlapping peptide synthesis indicated that the epitope was contained in the sequence HIPPENIFPLE. This sequence was unique to GCC, and despite a short stretch of homology with serum amyloid protein and pertussis toxin, no cross reactivity was detected. The core epitope was delineated using a random hexameric phage display library, and two categories of sequences were identified, containing either a single, or two adjacent proline residues. No sequence identified by phage display was identical to the epitope present in GCC, indicating that phage sequences represented mimotopes of the native epitope. Alignment of these sequences with HIPPENIFPLE suggested duplication of the recognition motif, which was confirmed by peptide synthesis. These studies allowed us not only to define the requirements of epitope recognition by GCC:B10 monoclonal antibody, but also to describe a novel means of epitope recognition involving topological mimicry and probable duplication of the cognate epitope in the native guanylyl cyclase C receptor sequence.

Keywords: epitope mapping; guanylyl cyclase C; monoclonal antibody; phage display

Membrane associated guanylyl cyclases serve as receptors for a variety of polypeptide hormones (Drewett & Garbers, 1994). These receptors have a large extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic catalytic domain that harbors guanylyl cyclase activity. Binding of specific ligands to these receptors results in an increase in the guanylyl cyclase activity of the receptor leading to elevated intracellular concentrations of cGMP (Chao et al., 1994). We have focused our attention for some time on a specific member of the guanylyl cyclase receptor family, guanylyl cyclase C. GCC serves as a receptor for the newly described hormones, guanylin and uroguanylin (Currie et al., 1992; Forte & Hamra, 1996), in addition to the heat-stable enterotoxin peptides (De Sauvage et al., 1991; Garbers, 1992). Binding of stable-toxin peptides to the extracellular domain of GCC receptor leads to cGMP-mediated signal transduction events

(Chao et al., 1994) culminating in a form of severe, transient watery diarrhea. In order to study the localization of GCC in individual regions of the normal mammalian intestine, we have generated a monoclonal antibody to the human receptor, which we call GCC:B10 (Nandi et al., 1997). A region of 63 amino acids was identified, present in the intracellular domain of GCC, which contained the epitope for GCC:B10 monoclonal antibody. No apparent sequence homology was observed between this stretch of 63 amino acids and any other protein in the sequence database, including the close members of the guanylyl cyclase receptor family. However, this region possessed considerable homology to the reported GCC isoforms cloned from various mammalian species (Nandi et al., 1997).

Recent reports implicate GCC as a potential marker for the diagnosis and surveillance of metastatic colon cancers (Carrithers et al., 1994, 1995, 1996). Expression of GCC in primary and metastatic colorectal tumors is reported to be similar to that in normal tissue. These studies were performed in membrane fractions prepared from colorectal carcinoma tissues, by monitoring

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stable toxin binding to these membranes (Carrithers et al., 1994, 1995, 1996). Extraintestinal expression of GCC was not detected except in tumor tissue originating from the metastasis of colorectal carcinoma. It therefore appears that GCC:B10 monoclonal antibody could be used for the detection of GCC in these tumors as a powerful diagnostic tool.

The use of monoclonal antibodies for the characterization and detection of biomolecules is largely determined by the specificity of interaction of the antibody with its recognition domain. In the case of proteins, peptides can mimic the epitope present in the native protein, either due to similarity in the amino acid sequence or in the conformation adopted by the peptide. When epitope sequences contain proline residues, it is likely that interaction with the antibody could be determined by a conformation induced by the presence of the proline residues (Feller et al., 1994; Yu et al., 1994). Therefore, monoclonal antibodies can accommodate a certain degree of flexibility in interaction provided that their recognition motifs are conformational or topological mimics of the native epitope.

Identification of epitopes for monoclonal antibodies by overlapping peptide synthesis has been well recognized as a useful tool to characterize interacting motifs. The advent of phage display technology has increased the repertoire that can be searched for reacting domains (Scott & Smith, 1990) and widened the scope of identification of structurally similar motifs adopted by dissimilar sequences. A library of phages is panned against a particular antibody and the DNA of individual phages is sequenced to determine the insert in the gpIII coat protein. In many cases, this has allowed the identification of epitope sequences not identical to that present in the protein antigen, thereby describing sequences that mimic the native epitope (Cha et al., 1996; Demangel et al., 1996; Pacholczyk & Sweadner, 1997). Phage display also provides information on the critical amino acids that are required for interaction with a specific antibody, and as a corollary, identifies amino acid replacements in the epitope that can be tolerated by the paratope of the monoclonal antibody in question. In view of the potential importance of GCC:B10 monoclonal antibody in performing immunohistochemical analysis of GCC expression in normal and cancerous tissues, we wished to ascertain that the antibody we have obtained is specific to this receptor, as well as establish the family of peptide sequences that it has the potential to recognize. We decided to identify the core epitope of GCC:B10 using both peptide synthesis and phage display techniques, and our results suggest an interesting mode of epitope recognition by this antibody, which could serve as a prototype for the study of topological mimics in future.

Results

Identification of the epitope for GCC:B10 by peptide synthesis

We have earlier reported that the epitope for GCC:B10 was contained in a stretch of 63 amino acids adjacent to the transmembrane span of the receptor, and interaction with the antibody was detected in fixed tissues and in denatured antigen, indicating that the epitope was likely to be sequential in nature (Nandi et al., 1997). To identify the minimal binding region of the monoclonal antibody, peptides corresponding to the entire stretch of 63 amino acids identified earlier to harbor the epitope for the monoclonal antibody were synthesized on polyethylene pins. The data shown in Figure 1A indi-

cate the reactivity of GCC:B10 with the individual peptides contained within the 63 amino acid stretch. A peptide with the sequence HIP-PENIFPLE, showed maximum cross reactivity. However, adjacent sequences QKKWSHIPEN and IFPLETNETNH also showed some cross reactivity. The peptide HIPPENIFPLE was synthesized and used to monitor reactivity with GCC:B10 in an ELISA using immobilized recombinant receptor fragment (GCC-ED5) harboring the epitopic sequence. As shown in Figure 1B, the peptide HIP-PENIFPLE was able to inhibit the binding of GCC:B10 to the receptor, indicating that the complete recognition motif for GCC:B10 was contained in the 11 amino acid peptide. The IC_{50} of binding, determined from the displacement curves was 2.1×10^{-7} M. The epitope sequence HIPPENIFPLE is present 15 amino acids after the transmembrane span of GCC (Fig. 1C).

Comparison of this 11-mer epitopic region with the close members of the guanylyl cyclase family revealed no sequence homology with either GCA or GCB (Table 1). This suggests that the monoclonal antibody GCC:B10 is specific to GCC. Comparison of the reported sequences for GCC isoforms from various species, showed maximal conservation of the epitope across the mammalian species, while the *Xenopus* sequence in the epitopic region varied considerably (Table 1).

Cross-reactivity of GCC:B10 monoclonal antibody with serum amyloid P precursor and pertussis toxin

A search of the protein sequence database to identify sequences with homology to the HIPPENIFPLE sequence indicated interesting sequence similarity with two proteins, namely serum amyloid P precursor and the A subunit of pertussis toxin (Fig. 2A). In order to test the cross reactivity of GCC:B10 with these proteins, we performed Western Blot analysis with GCC:B10, using purified serum amyloid protein and the A subunit of pertussis toxin. We did not observe any reactivity with either of these proteins and GCC:B10 (Fig. 2B). Similar results were obtained in an ELISA analysis (data not shown), indicating that certain constraints in the epitope sequence in GCC, brought in by adjacent residues, could determine the reactivity of the GCC:B10 monoclonal antibody.

Immunoselection of phages from a random phage display library

We decided to fine map the core epitope for GCC:B10 monoclonal antibody by phage display technology using a random pentavalent hexameric phage display library (Scott & Smith, 1990), with the assumption that the minimum recognition motif could be contained in a stretch of amino acids shorter than the 11-mer HIP-PENIFPLE sequence. GCC:B10 IgG was immobilized on ELISA strips and phages were applied to the wells. The complexity of the library used was 2×10^8 and concentration of the physical particles were 2×10^{14} /mL. Therefore in 2×10^{11} phages every sequence was represented at least 1,000 times. In early rounds of panning, phages were eluted with high concentrations of GCC:B10 IgG, but in later rounds of panning, the phages bound to immobilized GCC:B10 were eluted with the HIPPENIFPLE peptide (10^{-6} M) to ensure specificity in identifying homologous interacting motifs inserted in the phage coat protein.

To select phages to be sequenced after four rounds of biopanning, isolated colonies of K91Kan cells infected with phages were transferred to nitrocellulose filters. The filters were probed with

Fig. 1. Epitope mapping of GCC:B10 monoclonal antibody. **A:** 11-amino acid peptides were synthesized on polyethylene pins. The pins were incubated with 1 μ g/mL of GCC:B10 monoclonal antibody and bound antibody detected with anti-mouse IgG conjugated to alkaline phosphatase. Values represent the mean $\pm$ S.E. of three experiments, with each pin synthesized in duplicate. **B:** GCC-ED5 protein (100 ng/well) was immobilized on ELISA wells and incubated with HIPPENIFPLE peptide and 100 ng/mL of GCC:B10 monoclonal antibody. Bound antibody was detected using anti-mouse IgG conjugated to alkaline phosphatase and p-nitrophenyl phosphate as substrate. **C:** Location of the HIPPENIFPLE epitope in GCC receptor. TM: transmembrane domain of GCC; PKLD: protein kinase-like domain of GCC.

GCC:B10 monoclonal antibody as well as the antibody preincubated with HIPPENIFPLE peptide, and no reactivity was observed with any of the phages when GCC:B10 monoclonal antibody was preincubated with peptide, thereby confirming specificity of phage interaction (data not shown). Individual phages were isolated, DNA prepared, and the sequence of the hexapeptide inserted in the gpIII coat protein deduced from the DNA sequence. The data shown in Table 2 indicate the frequency of individual phages obtained with

a particular hexapeptide sequence. Most of the sequences contained two proline residues while two sequences contained a single proline residue. Also apparent is the high frequency of hydrophobic amino acids observed in the hexapeptide sequences, with residues such as leucine and valine occurring in all the identified peptide. It is interesting to note that none of the phage sequences shared any sequence identity to the native epitope HIPPENIFPLE, indicating that they were mimotopes of the native epitope.

Table 1. Alignment of sequences in the region of the HIPPENIFPLE epitope in various guanylyl cyclases and guanylyl cyclase C receptor isoforms cloned from various species

Guanylyl cyclase	Sequence	Acc.no. ^a
GCA	KELVSEL...WR.VRWEDLQPSSLERHLR	R47859
GCB	KELASML...WR.IRWEELQFGNSDRYHK	M26896
GCC	HELR...QKKWSHIPPENIFP...LETNET	M73489
Human GCC	HIPPENIFPLE	M73489
Porcine GCC	HIPPENIFPLE	D17513
Rat GCC	HIPSENIFPLE	M55636
Guinea pig GCC	HIPPEKILPLE	Z74734
Xenopus GCC	HISA EKILPLN	D49837

^aEMBL Gene Bank Accession Number.

Fig. 2. Reactivity of GCC:B10 monoclonal antibody with serum amyloid P precursor and pertussis toxin. **A:** Sequence homology between the HIP-PENIFPLE peptide and serum amyloid P precursor and pertussis toxin. **B:** Proteins were resolved by SDS gel electrophoresis and gel stained with Coomassie Blue R250 or proteins transferred to PVDF membranes and probed with GCC:B10 monoclonal antibody.

To confirm the reactivity of the phages, individual phage clones were immobilized on ELISA wells and uniform coating of each phage was monitored using a monoclonal antibody to the gpIII protein. The coating was comparable in all the wells (Fig. 3). The phages showed varying degrees of reactivity with the GCC:B10 monoclonal antibody in ELISA, but it was apparent that the phage PLVAPA bound the antibody to the maximum (Fig. 3). The variable reactivity of individual phages with GCC:B10 monoclonal antibody could be a reflection of the difference in the affinity of the individual phages to GCC:B10 monoclonal antibody. It is important to note that the reactivity of the epitope sequence was being evaluated in the context of the entire gpIII coat protein, since intact phages were immobilized.

Table 2. Alignment of phage peptide sequence mimotopes with the native epitope in GCC^a

	H	I	P	P	E	N	I	F	P	L	E	Frequency
G	L	L	P	P	A							4
G	L	L	P	P	F							1
G	L	L	P	P	G							1
D	L	L	P	P	F							6
D	L	L	P	P	S							7
A	L	L	P	P	S							1
					L	L	V	V	P	S		1
					P	L	V	A	P	A		2

^aSequences obtained after phage display are aligned in the two halves of the native epitope sequences. Highlighted residues are those common to the native epitope or within different phage sequences. The frequency of individual phages with a particular hexapeptide sequence obtained is indicated in the extreme right hand column. The amino acids to the N-terminus and the C-terminus of the hexapeptide sequence present in the gpIII coat proteins are alanine and proline, respectively.

Fig. 3. Reactivity of isolated phages with GCC:B10 monoclonal antibody and anti-gpIII antibody. Phages were immobilized on an ELISA plate and incubated with GCC:B10 antibody or with gpIII antibody. Bound antibodies were detected using anti-mouse horseradish peroxidase. Values represent the mean $\pm$ S.E. of duplicate determinations with each assay performed thrice. Inset indicates the reactivity of individual phages following Western blot analysis and probing of the membrane with GCC:B10 antibody.

Western blotting was performed with phages representing different clones and GCC:B10 monoclonal antibody (Fig. 3, inset). Reactivity was registered with all the phages to varying degrees. However, the phage containing the sequence PLVAPA did not show any detectable cross reactivity. Therefore it can be hypothesized that a structural constraint in conjunction with a sequence is necessary to maintain the epitope cross reactivity of the PLVAPA sequence.

Mimotope identification by phage display

Peptides corresponding to some of the sequences present in the phages, and which were represented infrequently, were synthesized on polyethylene pins, to show specificity of GCC:B10 monoclonal antibody binding to these sequences (Fig. 4). Reactivity with all the sequences was observed, albeit to varying extents. It was observed that single proline residue containing peptides reacted less well than the two-proline residue containing peptides. This is in contrast to the results shown in Figure 3, where presentation of the PLVAPA sequence in the context of the gpIII protein allowed maximum reactivity with the GCC:B10 monoclonal antibody, again emphasizing the role of structural features in allowing antibody reactivity.

Of the sequences containing two proline residues, more than half contained an aspartate residue at the N-terminus, indicating that interaction with GCC:B10 was enhanced by an acidic residue in the hexapeptide sequence. The native epitope HIP-PENIFPLE also contained two glutamate residues, and as will be apparent later, the presence of a single acidic residue is sufficient to bring about interaction with GCC:B10 antibody. A variety of amino acids are tolerated at the C-terminus of the hexapeptide epitope, indicating that this residue may be least critical for paratope interaction. Therefore a broad consensus sequence for the epitopes identified with two proline residues could be (D/G/A)LLPP(X).

Fig. 4. Reactivity of peptide sequences identified by phage display. Peptides with the indicated sequences were synthesized in duplicate on polyethylene pins and incubated with GCC:B10 monoclonal antibody. Bound antibody was detected using anti-mouse IgG conjugated to alkaline phosphatase. Values represent the mean $\pm$ S.E. of three experiments, with each pin synthesized in duplicate.

Alignment of the two sequences containing a single proline residue proved to be problematic since the sequences did not fit into the consensus described above. While these two phages were isolated relatively infrequently, good cross reactivity with GCC:B10 indicated that they were specific in their interaction with the antibody. On scrutiny of the native epitope sequence, it became apparent that the later half of the epitope, namely ENIFPLE, contained a single proline residue, as well as an acidic residue which seemed to be necessary for antibody reactivity, and it was now possible to align the peptide sequences containing a single proline residue with this region of the epitope, as shown in Table 2. What this implies is that the core epitope for recognition by GCC:B10 monoclonal antibody was repeated contiguously in the native receptor sequence, with duplication involving a subtle topological mimicry. We, therefore, hypothesized that within the 11-mer HIPPENIFPLE sequence, were two *independent* recognition motifs for GCC:B10, one represented by the sequence HIPPEN and the other perhaps by ENIFPLE (Table 2). The hypothesis was strengthened by the fact that in the original synthesis of 11-amino acid overlapping peptides (Fig. 1) some peptides showed reactivity to GCC:B10 which were adjacent to the highly immunoreactive HIPPENIFPLE sequence.

Epitope duplication in the GCC sequence

In order to confirm that individual half-sequences of the original 11-mer epitope were independent interacting regions, peptides were synthesized with the sequences WSHIPPEN and ENIFPLET on polyethylene pins and reactivity tested with the monoclonal antibody. The sequences synthesized maintained a length of 8 amino acids which were possibly required for interaction of the monoclonal antibody with the peptide epitope when synthesized directly on the pins, and contained the sequences which we hypothesized were independent epitopes. As shown in Figure 5A, interaction was observed with both the peptides with GCC:B10 monoclonal antibody. Moreover, randomized peptide sequences with amino acid composition identical to the native peptide sequence did not show any reactivity with the monoclonal antibody. This validated our hypothesis of independent reactivity of the two halves of the native HIPPENIFPLE peptide.

Fig. 5. Interaction of HIPPEN and ENIFPLE peptides with GCC:B10 monoclonal antibody. **A:** Peptides with the indicated sequences were synthesized on polyethylene pins in duplicate and reacted with GCC:B10 monoclonal antibody. As controls, randomized sequences were also synthesized and analyzed for their reactivity with the antibody. Values represent the mean $\pm$ S.E. of three experiments. **B:** Inhibition of binding of GCC:B10 antibody to HIPPENIFPLE peptide by HIPPEN and ENIFPLE peptides. Varying concentrations of HIPPEN and ENIFPLE peptides were incubated individually with GCC:B10 antibody in ELISA wells coated with HIPPENIFPLE peptide. Bound antibody was detected using anti-mouse IgG conjugated to horseradish peroxidase, and the data obtained are representative of one experiment, with each analysis repeated thrice.

In order to determine if peptides HIPPEN and ENIFPLE had a similar affinity to GCC:B10 antibody, as the native 11-mer peptide, individual peptides of sequence HIPPEN and ENIFPLE were synthesized, purified, and used in an ELISA to inhibit GCC:B10 binding to HIPPENIFPLE peptide. As shown in Figure 5B, both the peptides bound the monoclonal antibody with comparable affinities, and with an affinity similar to HIPPENIFPLE (Fig. 1B). This implied that the critical determinants for epitope recognition by GCC:B10 were present in both HIPPEN and ENIFPLE.

The experiments described above suggest that there could be two possibilities for the mode of interaction of the antibody with the epitope peptides: (1) the binding pocket is composed of a multiple interacting site with the HIPPENIFPLE peptide, and that the two halves can be accommodated simultaneously; or (2) that a single binding pocket can accommodate any of the peptides and binding is competitive, as depicted in Figure 6A. To test these two possibilities, each individual peptide, namely, HIPPENIFPLE, HIPPEN, and ENIFPLE, was immobilized on an ELISA plate and binding of GCC:B10 tested in the presence of any of the other peptides. The results depicted in Figure 6B indicate that either HIPPEN or ENIFPLE could inhibit binding of GCC:B10 to the full

Fig. 6. Interaction of GCC:B10 monoclonal antibody with the peptide epitopes. **A:** Proposed models for the interaction of GCC:B10 monoclonal antibody with the peptide epitopes. **B:** Competitive inhibition of binding of GCC:B10 monoclonal antibody to peptides HIPPENIFPLE, HIPPEN, and ENIFPLE. Individual peptides were immobilized on ELISA plates and incubated with GCC:B10 antibody either in the presence or the absence of any of the three peptides. Bound antibody was detected by anti-mouse horseradish peroxidase, and values represent the mean $\pm$ S.E. of three experiments with each assay performed in duplicate.

length 11-mer HIPPENIFPLE peptide, as well as to each other, implying that a single binding pocket in the monoclonal antibody could accommodate all three peptides with comparable affinity. Therefore, epitope duplication of topological mimotopes had occurred in the primary sequence of GCC, and this could be advantageous in increasing the binding of GCC:B10 monoclonal antibody to the receptor in immunohistochemical analysis.

Discussion

Our studies reported here using peptide synthesis coupled to phage display, as a means to identify the epitope for a GCC monoclonal antibody, provide a comparison of the efficacy of the two most popular epitope-mapping techniques. The minimal region of interaction of GCC:B10 in GCC was contained in the sequence HIPPENIFPLE, as detected by peptide synthesis, but phage display analysis revealed duplication of the recognition motif in the receptor sequence. The synthetic peptide with the sequence HIPPENIFPLE was able to completely inhibit the binding of the monoclonal antibody to the GCC-ED5 protein suggesting that the critical recognition domain of the epitope is contained in the peptide HIPPENIFPLE. Therefore, selective replacements of amino acids in the epitope could identify residues involved in direct interaction with the monoclonal antibody, but peptides identified by phage display suggested a critical requirement for proline residues, indicating that the turn generated by the proline residue could "fit" into the antibody paratope conformation. It is also interesting to

note the presence of a large number of hydrophobic amino acids in the epitope sequence, such as I, F, and L. This suggests that the surface of the epitope, when combined with GCC:B10, could be altered from that occurring in the absence of the antibody (Geysen et al., 1988). Therefore, the final structure adopted by the antigen when complexed with the antibody could be distinct to that observed in the receptor, supporting the "induced fit" concept of antigen-antibody interaction. We are presently attempting to obtain crystallographic data of GCC:B10 monoclonal antibody-peptide complexes, and these data should verify the conclusions that we have drawn in this present study.

Comparison of the epitope sequence with sequences present in the Protein Data Bank (PDB) identified serum amyloid protein (Emsley et al., 1994) and the A subunit of the pertussis toxin (Stein et al., 1994) proteins containing partial homology with the HIPPENIFPLE sequence. However, neither on Western blotting or in ELISA could any reactivity of the GCC:B10 with these proteins be detected thereby indicating conformational constraints associated with the epitope. The epitope homology sequence in serum amyloid protein is located in the junction of the α -helix and β -sheet where the proline residues are present (Emsley et al., 1994). Therefore it can be presumed that the presentation of the structural determinant is not conducive for antibody reactivity. Similarly, the proline rich region in the pertussis toxin is flanked by charged arginine residues, which could inhibit binding of pertussis toxin to GCC:B10 by unfavorable charge interactions (Stein et al., 1994).

We have provided sufficient evidence that the sequences that have been identified (Table 2) do indeed interact with the GCC:B10 monoclonal antibody. What is intriguing is that phage display did not lead to the isolation of any phages that harbored sequences in the gpIII protein that were identical to the native epitope. Sequences identical to the native epitope may not have been isolated if the affinity of the phage-displayed sequences in the context of the gpIII coat protein were higher than that of the native epitope. It is also possible that the native sequence when expressed in the entire phage could be toxic and therefore selected against, or the complexity of the library was not sufficient to isolate identical sequences. Whatever the case, our experiments clearly led to the identification of mimotopes for GCC:B10 monoclonal antibody.

The importance of the proline residue in both the native and mimotope sequence indicates the requirement of a turn motif in the region of the peptide that interacts with GCC:B10. It is also interesting to note that a large number of hydrophobic amino acids are present in both the native epitope and mimotope sequences. As has been indicated earlier, a large number of mimotopes identified by various investigators earlier, contain predominantly proline, glycine, and hydrophobic residues (Oldenburg et al., 1992; Scott et al., 1992; Hoess et al., 1993). In many cases the mimotopes identified were against antibodies directed to nonpeptide antigens, such as carbohydrates and biotin. However, there is one example published recently where a mimotope for a monoclonal antibody to the Na,K-ATPase also contained proline residues and other hydrophobic amino acids (Pacholczyk & Sweadner, 1997). Whether this is a result of common modes of interaction with paratope sequences, or represents the greater flexibility allowed in hydrophobic interactions with a variety of amino acids in the paratopes of different monoclonal antibodies, will be known only after structural details are available.

The results that we have obtained using phage display methodologies strongly suggest to us that epitope duplication had occurred in GCC. However, another possibility could also be considered,

whereby the entire HIPPENIFPLE sequence provides significant contact with the antibody binding site, and that the shorter sequences HIPPEN and ENIFPLE could therefore inhibit binding to GCC:B10 of the longer peptide, and each other (Fig. 6B). There are no clear experiments that can be done to conclusively prove either hypothesis, except determination of the structure of the monoclonal antibody complexed with the individual peptides. What strengthens our hypothesis, however, was the fact that we were able to narrow down the epitopic sequences to a core of only four amino acids using phage display (Table 2), indicating that perhaps only a small region of the HIPPENIFPLE sequence was required for antibody recognition, thereby allowing duplication of the recognition motif in the original 11-amino acid stretch. Indeed, if a larger sequence with multiple interactions involving many amino acids, as defined by HIPPENIFPLE, was required for antibody interaction, we may never have obtained interacting phages which provided an efficient binding epitope for the monoclonal antibody, using a hexameric phage display library. As indicated earlier, the only conclusive evidence validating either hypothesis can be obtained from X-ray crystal structures, and such studies are underway.

While epitopes repeated contiguously in proteins have been described, the sequences that were reported earlier were nearly identical (Fontenot et al., 1995; Stoute et al., 1995). However, epitope recognition by GCC:B10 accommodates some degree of flexibility in the structural features that the antibody identifies (Smith et al., 1995), resulting in quite dissimilar sequences allowing interaction with GCC:B10. As we have seen in this study, a single binding pocket in the antibody could accommodate any of the epitope peptide sequences, and therefore binding to one peptide sequence could be inhibited by the other sequence. While this degree of flexibility in the recognition of various epitopes may appear to be contrary to what one would wish for in an antibody used for immunohistochemical analysis, it is quite clear from our results that specificity is also engineered in the paratope of GCC:B10. This specificity in the interacting epitope of GCC:B10 makes it ideally suitable for immunohistochemical analysis of colorectal tissue. Furthermore, duplication of the epitope in a contiguous manner may allow the presentation of the epitope in a "concentrated" form allowing more efficient interaction of the antibody with the antigen. This could perhaps explain the good reactivity observed in immunohistochemical analysis that we have observed with the GCC:B10 antibody (Nandi et al., 1997), despite the apparent low affinity of interaction of the peptide to the monoclonal antibody. Our results presented here strongly advocate the use of this antibody in the analysis of colorectal tumors and such studies are underway in the laboratory.

Materials and methods

Synthesis of peptides on polyethylene pins

Peptide synthesis and epitope mapping were carried out by the Geysen technique (Geysen et al., 1984, 1987) using F-moc chemistry. A 96-well format peptide synthesis kit (Cambridge Research Biochemicals, UK) was used. Peptides, 11 amino acid in length, corresponding to the entire 63 amino acid stretch containing the epitope for GCC, were synthesized in duplicate on polyethylene pins, with an overlap of 5 amino acids. ELISA confirmed reactivity of the antibody with the synthesized peptides. ELISA was carried out by treating both the pins and the ELISA plates with phosphate buffer saline (PBS), pH 7.2 containing 2% bovine serum albumin.

GCC:B10 monoclonal antibody (150 μ L of a 1 μ g/mL solution) was added to each well of an ELISA plate, and the pins were submerged in the solution. Following 3 h incubation with the monoclonal antibody the pins were washed with PBS containing 0.1% Tween 20 and incubated with anti-mouse IgG conjugated to alkaline phosphatase (Gibco/BRL). Following a further 2 h incubation, the pins were washed with PBS containing 0.1% Tween 20 and color developed using p-nitrophenyl phosphate (1 mg/mL) prepared in 0.1 M diethanolamine buffer pH 9.8. The reaction was terminated using 1N NaOH and the absorbance was recorded at 405 nm in an ELISA reader (7520 Microplate Reader, Cambridge Technology, Inc.).

Purification of synthetic peptides

The 11-amino acid peptide HIPPENIFPLE, was synthesized by PeptideGenic Research and Co. (Livermore, California). Typically 10 mg of the peptide was weighed every time and resuspended in 0.5 mL distilled water and purified by reverse-phase HPLC, using a Shimadzu HPLC system (Shimadzu Analytical Instruments, Japan) in a Shim-pack prep-ODS(H) preparative column (20 mm $\times$ 250 mm). The peptide was bound to the column in a buffer A (containing 0.05% CF₃COOH in water) and the bound peptide eluted using a gradient of 0% to 100% of buffer B (containing 70% acetonitrile) at a flow rate of 5 mL/min. Fractions were analyzed using an analytical column Shim-pack ODS(H) (4.6 mm $\times$ 250 mm), with a gradient of 30% to 70% of buffer B in 45 min at a flow rate of 0.5 mL/min. The peptide of interest eluted with a retention time of 23.4 min.

Peptides HIPPEN and ENIFPLE were solubilized in 25% ammonia solution. The peptides were purified by reverse-phase HPLC, on a Shim-pack prep-ODS(H) preparative column. The peptides were bound to the matrix in a buffer A (containing 0.05% CF₃COOH in water), and the bound peptides eluted using a gradient of 10% to 100% of buffer B (containing 80% acetonitrile and 0.05% CF₃COOH in water) over 60 min at a flow rate of 5 mL/min. Fractions were collected and analyzed as described above. The HIPPEN peptide eluted with a retention time of 21.8 min while the ENIFPLE peptide eluted with a retention time of 33.06 min. All peptides were quantitated by amino acid analysis as described earlier (Cohen & Strydom, 1988).

ELISA with GCC-ED5 and synthetic peptide epitopes

ELISA plates (Nunc, Denmark) were coated with purified GCC-ED5, a region of the receptor shown in our earlier studies to encompass the epitope for GCC:B10. The plates were incubated for 16 h at 4°C, washed and blocked in PBS containing 0.2% bovine serum albumin. The wells were then incubated for 2 h with 25 ng/mL of GCC:B10 monoclonal antibody diluted in 50 μ L of PBS containing 0.1% Tween 20, 0.2% BSA and varying concentrations of HIPPENIFPLE peptide. Bound antibody was detected using an anti-mouse horseradish peroxidase conjugate (Amersham, UK), and 3,3',5,5'-tetramethyl benzidine (Bangalore Genei) as the substrate. The reaction was terminated using 50 μ L of 3 N H₂SO₄ and the absorbance was recorded at 450 nm. The data were analyzed using GraphPad Prism software (San Diego, California).

The peptides HIPPEN and ENIFPLE were purified, quantitated as described above, and used in similar inhibition ELISAs. HIPPENIFPLE peptide was coated at a concentration of 100 ng/well. The plates were incubated for 16 h at 4°C, washed and blocked in PBS containing 0.2% bovine serum albumin. The wells were then

incubated for 2 h with 25 ng/mL of GCC:B10 monoclonal antibody diluted in 50 μ L PBS containing 0.1% Tween 20, 0.2% BSA and varying concentrations of either HIPPEN or ENIFPLE peptide. Bound antibody was detected using an anti-mouse horseradish peroxidase conjugate.

Coupling of HIPPENIFPLE, HIPPEN, and ENIFPLE peptides to covalink NH plates

Since the peptides HIPPEN and ENIFPLE did not adsorb efficiently to regular ELISA plates, these peptides were immobilized on Covalink NH plates (Nunc, Denmark). Peptide-NHS solution was prepared fresh in water (6 μ g/mL of each of the peptide was mixed with 0.184 mg of Sulfo-NHS in a reaction volume of 1 mL) and 100 μ L of the solution was added per well. Conjugation was mediated by addition of 50 μ L of EDC solution prepared in distilled water and the reaction allowed to proceed at room temperature for 2 h. The wells were washed with Cova buffer (PBS containing 0.5% Tween 20, 2 M NaCl and 40 mM MgSO₄). The wells were blocked with 0.2% BSA and ELISA was performed as described earlier.

Cross reactivity of GCC:B10 monoclonal antibody with serum amyloid P precursor and pertussis toxin

Pertussis toxin (Sigma) and serum amyloid protein (a kind gift from Prof. N.H.H. Heegaard, Statens Serum Institute, Denmark) were resolved on a 12% SDS-polyacrylamide gel. The proteins were transferred to PVDF (Millipore, Bedford, Massachusetts) membranes in Tris-glycine buffer, pH 8.3, containing 20% methanol, membrane blocked with 5% enhanced chemiluminescence (ECL) blocking solution (Amersham, UK) and then incubated with GCC:B10 monoclonal antibody (1 μ g/mL diluted in PBS containing 0.2% BSA and 0.1% Tween 20) for 2 h at room temperature. Membranes were washed and incubated with anti-mouse IgG conjugated to horseradish peroxidase (1:2500 diluted in PBS containing 0.2% BSA and 0.1% Tween 20) for 1 h and the bound antibody detected by ECL (Amersham) following exposure of the membrane to X-ray film as per manufacturer's instructions.

Immunoselection of GCC:B10 reactive phages from a random, pentavalent, hexameric phage display library

The phage display library, constructed in fUSE 5 vector, was generously provided by Prof. G.P. Smith, University of Missouri, Columbia. Biopanning and sequencing were performed as reported earlier (Smith & Scott, 1993) with modifications as described below. GCC:B10 purified to homogeneity using protein A Sepharose chromatography was dialyzed against 100 mM NaHCO₃ buffer pH 8.6. A solution of GCC:B10 monoclonal antibody (100 μ g/mL, 100 μ L) was added to two wells in a eight-well polystyrene strips (Nunc) and the strip was incubated for 16 h at 4°C. The unbound protein solution was discarded and the wells were washed with 50 mM Tris Cl buffer pH 7.5 containing 150 mM NaCl (TBS). The wells were blocked with TBS containing 5 mg/mL of BSA and incubated for 1 h at room temperature. The wells were filled with the washing solution (50 mM Tris Cl buffer pH 7.5 containing 150 mM NaCl and 0.1% Tween 20), shaken for 1 min and contents of the well discarded. Washing was repeated six times, following which 2×10^{11} phages were diluted to 200 μ L in TBS-T buffer and 100 μ L added to each well coated with GCC:B10

monoclonal antibody. The wells were incubated at room temperature for 1 h, the unbound phage solution was discarded, and the wells were washed six times as described above. The bound phages were eluted with GCC:B10 monoclonal antibody (100 μ g/mL) in rounds 1 and 2 of panning and amplified phage obtained from the previous round of panning used in subsequent rounds. In rounds 3 and 4, the bound phages were first eluted with 100 μ L of 10^{-6} M solution of HIPPENIFPLE peptide in TBS.

Western blot analysis with GCC:B10-reactive phage particles

Phage particles (10^{11}), purified by polyethylene glycol 8000 precipitation of the culture filtrate of Kan91 cells infected with individual phages, were titered spectroscopically (Smith & Scott, 1993). Purified phages were boiled in SDS-PAGE sample buffer, pH 6.8 and resolved on a 12% SDS-polyacrylamide gel. The proteins were transferred to PVDF (Millipore, Bedford, Massachusetts) membrane in Tris-Glycine buffer, pH 8.3, containing 20% methanol, membrane blocked with 5% enhanced chemiluminescence (ECL) blocking solution (Amersham, UK) and then incubated with 1 μ g/mL of GCC:B10 monoclonal antibody (diluted in PBS containing 0.2% BSA and 0.1% Tween 20) for 2 h at room temperature. Membranes were washed and incubated with anti-mouse IgG conjugated to horseradish peroxidase (1:2500 diluted in PBS containing 0.2% BSA and 0.1% Tween 20) for 1 h and the bound antibody detected by ECL.

ELISA with GCC:B10 reactive phages

Phages (10^{10} /well) were coated to an ELISA plate in PBS for 16 h at 4°C. Wells were washed, blocked with BSA (5 mg/mL in PBS) for 1 h and reacted with GCC:B10 monoclonal antibody (5 μ g/mL) diluted in PBS containing 0.2% BSA and 0.1% Tween 20 for 2 h at room temperature. The wells were washed with PBS containing 0.1% Tween 20 and further incubated with anti-mouse IgG conjugated to horseradish peroxidase (1:1000 diluted in PBS containing 0.2% BSA and 0.1% Tween 20) for 1 h. The wells were washed and the bound antibody was detected using 3,3',5,5'-tetramethyl benzidine as the substrate. The absorbance was recorded at 450 nm. To confirm the coating of the phages to the ELISA plate, a monoclonal antibody to gpIII (Bhardwaj et al., 1995) which was a kind gift from Dr. Vijay K. Chaudhary, University of Delhi, was used at a dilution of 1:50,000 in an assay similar to that described above.

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