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Antigenic determinants on chicken riboflavin carrier protein. A study with monoclonal antibodies

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Abstract. Monoclonal antibodies raised against chicken egg white riboflavin carrier protein were classified into seven categories each recognizing a distinct epitope. Of these, six were directed against conformation dependent epitopes and one to a sequential epitope. The roles of lysine residues and the post-translationally attached phosphate and oligosaccharide moieties in the antigenicity of riboflavin carrier protein recognized by the monoclonal antibodies were investigated. The binding region of three monoclonal antibodies could be located within the 87–219 amino acid sequence of the protein and one antibody among these recognized a sequence of 182–204 amino acid residues. All the monoclonal antibodies were able to recognize riboflavin carrier proteins present in the sera of pregnant rats, cows and humans indicating that the epitopes to which they are directed are conserved through evolution from chicken to the human.

Keywords. Riboflavin carrier protein; antigenic determinants; monoclonal antibodies.

1. Introduction

Riboflavin carrier- (or binding-) protein (RCP), first isolated and characterized from the chicken egg white and yolk, is highly conserved both physicochemically and functionally through evolution from birds to mammals including primates (Adiga *et al* 1991). One of its remarkable characteristics is its immunogenicity potential as a hetero-antigen in most animal species hitherto tested. This indicates that while retaining basic structural features essential for biological activities (such as riboflavin binding and subsequent carrier function for embryonic vitamin nutrition), RCP has undergone subtle changes at discrete regions during evolution such that it can be recognized as non-self and elicit high titre antibody production. We raised monoclonal antibodies (MAbs) to chicken egg white RCP and used them as specific probes for delineating the nature of these antigenic determinants. Preliminary characterization of 3 of these MAbs has been reported earlier (Visweswariah *et al* 1987). Since theoretical considerations of the antigenic profile deduced from the amino acid sequence of RCP indicated the probability of more than 3 epitopes on its surface, the production and characterization of additional MAbs was considered essential to understand the overall antigenic profile of this vitamin carrier.

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Abbreviations used: RCP, Riboflavin carrier protein; PBS, phosphate buffered saline 150 mM NaCl, 10 mM sodium phosphate (pH 7·4); MAb, monoclonal antibody; FPLC, fast protein liquid chromatography; SPRIA, solid phase radioimmunoassay; LPRIA, liquid phase radioimmunoassay.

Furthermore, since immunoneutralization of RCP interferes with its function in embryonic growth in mammals (Adiga *et al* 1991) it is of interest to determine whether specific MAbs could be generated to various biologically important regions of the protein such as those involved in ligand-binding and in recognition of putative receptor sites implicated in transport across the oocyte/placental membrane barriers.

Earlier studies using polyclonal antisera and three MAbs to chicken RCP have indicated immunological cross-reactivity among RCPs. The data presented in this communication deal with the extensive characterization of six conformation-specific MAbs with emphasis on the conservation of the corresponding epitopes on RCP in species other than chicken. Further, attempts have been made to identify certain specific segments on the RCP where the epitopes recognized by some of these MAbs are localized.

2. Materials and methods

2.1 *Purification/enrichment of RCPs*

The purification of RCP from the chicken egg white, yolk and the plasma from estrogenized chicken, was carried out as outlined earlier (Murthy and Adiga 1977). To obtain apo-form of RCP, to the protein dissolved in 50 mM acetate buffer, pH 3·5, dextran coated charcoal was added, mixed thoroughly and centrifuged to remove riboflavin bound to charcoal. Enrichment of RCP present in the sera of pregnant rat, cow and human was carried out as reported earlier (Visweswariah *et al* 1987).

2.2 *Production and characterization of MAbs to chicken egg white apo-RCP*

The procedure employed for establishing MAbs was similar to that previously reported (Visweswariah *et al* 1987). Monoclonality of the specific antibodies was established by the elution pattern of [^{125}I]labelled RCP-antibody complex on a Superose-12 gel filtration column connected to the fast protein liquid chromatography (FPLC) system (Karande *et al* 1987) and confirmed by isotyping, employing specific antisera to either mouse *K* or chains or the various IgG subclasses (Visweswariah *et al* 1987). Association constants of the MAbs were determined according to Muller (1983).

2.3 *Growing of hybrid cells in serum free medium and preparation of* [^{125}*I*] *labeled MAbs*

Confluent hybridoma cultures were supplemented with Iscove's modified Dulbecco's medium (GIBCO) containing 0·5% BSA (tissue culture grade, Sigma) and 10 μg/ml transferrin (Cole *et al* 1985). After 48 h at 37° C, the culture supernatants were harvested and subjected to ammonium sulphate precipitation (50% saturation at 0°C). The precipitated IgG was dissolved in 10 mM $NaPO_4$ buffer (pH 7·4) and dialysed extensively against the same buffer. IgG (5 μg) was

labelled with 250 μCi of carrier-free [Na ^{125}I] (Amersham) by the iodogen method (Fraker and Speck 1978). The specific activity obtained was found to be 15–20,000 cpm/ng protein.

2.4 *Epitope analysis of monoclonal antibodies*

Characterization of the individual MAbs based upon their epitope identity was carried out by solid phase competition assay described by Stahli *et al* (1983) as well as by the Superose gel filtration method (Karande *et al* 1987).

2.5 *Covalent and non-covalent modifications of egg white RCP*

Denaturation with SDS or SDS plus ß-mercaptoethanol (ß-ME), reduction and carboxymethylation, succinylation (Visweswariah 1987) and dephosphorylation (White and Merrill 1988) of the antigen have been reported earlier. RCP deglycosylated by treatment with hydrofluoric acid (HF) (Manjunath and Sairam 1988) was a kind gift from Dr M R Sairam (Clinical Research Institute of Montreal, Canada) and was devoid of small molecular weight (M_r) fragments as assessed by SDS-PAGE.

2.6 *Digestion of egg white RCP with formic acid*

The experimental procedure adopted was that of Landon (1977). RCP (0·5–10 mg/ml) incubated with 75% (v/v) formic acid containing 7 M GuHCl at 37° C for 48 h was subsequently diluted 50-fold with water and lyophilized. The lyophilized material was dissolved and dialysed extensively against distilled water. In control experiments, RCP was treated individually with either GuHCl or formic acid. All three fractions were subjected to 8–25% gradient SDS-PAGE (Fling and Gregerson 1986) and the digestion products were visualized by exposing the SDS-gel to 4 M sodium acetate (Higgins and Dahmus 1979). The peptide-bound phosphate was stained on the gel (Bochner *et al* 1981). The gel fragments containing the resolved peptide bands were extracted with 0·05 M Tris-HCl buffer (pH 7·9) containing 0·l% (w/v) SDS, 0·l mM EDTA, 5 mM dithiothreitol and 0·15 M NaCl (Hager and Burgess 1980) and the peptides precipitated with acetone. A portion of the precipitated polypeptide fraction (molecular mass 25 K) was subjected to four cycles of Chang's manual microsequencing (Chang 1983) and the remaining was dissolved in PBS. The antigenicity of the individual polypeptides dissolved in PBS was analysed by solid phase radioimmunoassay (SPRIA) using the panel of MAbs.

2.7 *Isolation of phosphopeptide |His^{182}-Lys^{204}| from the tryptic digest of the egg white RCP*

Reduced and carboxymethylated RCP (RCM-RCP) (5 mg/ml) in 1% ammonium bicarbonate (pH 8·l) was digested with TPCK-trypsin at a ratio of l:50 of enzyme : substrate for 8 h at 30° C. To the clarified tryptic digest in water (5 mg/ml, pH 4·7), $BaCl_2$ was added to a final concentration of 0·25% (w/v), and treated with

an equal volume of ethanol; the precipitated phosphopeptide recovered by centrifugation, was dried with acetone and ether (Mansor and Annan 1971). The peptide was further purified by dissolving in water at pH 2·0; the precipitate was discarded and pH of the supernatant raised to 3·5 with 1 M NaOH. The phosphopeptide was recovered by precipitation with an equal volume of acetone. The purity of the peptide preparation was ascertained by 2D peptide map in cellulose coated thin layer plates (Chang 1983). The air-dried thin layer plate was sprayed first with ninhydrin reagent to visualize the peptide, and then exposed to the acid-molybdate reagent to reveal the peptide bound phosphate residues (Bochner *et al* 1981). The N-terminal sequence of this peptide was determined by Chang's method (1983).

2.8 *Immunoassays*

SPRIA and enzyme linked immunosorbent assay (ELISA) were performed by incubating the hybridoma culture supernatants with RCP (either native or chemically-modified) coated to polystyrene plates (Nunc), at a concentration of 5–10 μg/ml in PBS. Antibodies bound to immobilized RCP were detected either by addition of anti-mouse IgG conjugated to alkaline phosphatase (Sigma) or by quantifying bound [^{125}I] labelled protein A added subsequent to incubation with rabbit anti-mouse IgG (Visweswariah *et al* 1987).

Liquid phase radioimmunoassay (LPRIA) was performed as described (Visweswariah *et al* 1987). To determine inhibition with various samples, the concentration of MAb capable of binding 25-30% of input radioactivity was used for the assay. Radiolabelling of RCP was carried out by the iodogen method (Fraker and Speck 1978).

To compare the binding of [^{125}I]labelled chicken egg white, plasma and egg yolk RCPs by the different MAbs, the tracer (about 80,000 cpm, ~4 ng protein in each case) was incubated with individual MAU culture supernatants (undiluted) for 6 h at 37° C. The antibody bound radioactivity was precipitated with 1 µl of normal mouse serum and 25 µl of antiserum raised in rabbit to mouse IgG. The precipitate was dissolved in electrophoresis sample buffer and subjected to SDS-PAGE (Laemmli 1970). After drying the gel, it was exposed to an X-ray film at – 70°C for 12 h, and developed subsequently.

3. Results

By the immunization protocol followed for splenocyte fusion with SP2/0 cell-line, we obtained eighteen stable antibody secreting monoclonals specific to chicken egg white RCP. However, none of these MAbs could distinguish between apo- or holo-forms of egg white RCP in ELISA/LPRIA (data not shown), which is in line with the earlier report that the flavin binding site of egg white RCP is non-antigenic (Ramanathan *et al* 1980). These MAbs, including the 3 MAbs reported earlier (Visweswariah *et al* 1987), were subjected to epitope analysis by competitive inhibition of binding of [^{125}I] labelled MAbs to egg white RCP in the presence of individual unlabelled blocking MAbs in solid phase binding assay (Stahli *et al* 1983). Based upon the inhibition pattern, a total of *7* distinct reaction patterns that

reflected 7 individual epitopes well obtained (table l). While 6 of these MAbs recognize spatially well separated epitopes, 5A2E6 and 5BID3 MAbs seem to bind to overlapping epitopes.

Table 1. Checquer board analysis of inhibition of [^{125}I]labelled MAbs binding to RCP by unlabelled MAbs.

	[^{125}I]labelled MAbs						
Unlabelled MAbs	5B1D3	6H10F7	6A4D7	5G1E11	5A2E6	5C4C6	6B2C12
5B1D3 (3)	+	−	−	−	±	−	−
6H10F7 (4)	−	+	−	−	−	−	−
6A4D7 (3)	−	−	+	−	−	−	−
5G1E11 (2)	−	−	−	+	−	−	−
5A2E6 (2)	−	−	−	−	+	−	−
5C4C6 (2)	−	−	−	−	−	+	−
6B2C12 (2)	−	−	−	−	−	−	+

'+', '—' and `±' indicate inhibition, no inhibition and partial inhibition of binding of [^{125}I]labelled MAb.

The numbers in parentheses indicate number of MAbs belonging to the same group.

Varying amounts (50-200 ng) of RCP was adsorbed onto microtitre plates for 1 h at 37°C. Unoccupied sites were blocked with BSA. Unlabelled first antibody (0·05 ml) was added and incubated in the well for 4 h at 37° C. After washing, [^{125}I] labelled second antibody was added and incubated for 4h. The bound radioactivity after extraction with 3 N NaOH was measured in a gamma ray spectrometer.

For all subsequent experiments one representative from each of these 7 MAb groups was chosen and these were represented as 5BID3, 6A4D7, 6B2C12, 6Hl0F7, 5A2E6, 5GIEll and 5C4C6. The K_a, values for all the above MAbs as determined by the method of Muller (1983) ranged from 10^{-7} to 10^{-10} M (table 2).

Table 2. IgG isotypes and affinity constants of RCP monoclonal antibodies.

Monoclonal antibody	IgG subclass	Affinity constant K_a 10^9 M^{-1}
5B1D3	IgG2a, K	30
6H10F7	IgG1, K	0·3
6A4D7	IgG2b, K	1·3
5C4C6	IgG1, K	4·3
5A2E6	IgG2a, K	1·3
5G1E11	IgG1, K	0·18
6B2C12	IgG2a, K	0·03

IgG subclass was determined in SPRIA using anti mouse heavy chain and light chain subclass specific antibodies. Affinity constant was determined in competitive LPRIA according to Muller (1983).

3.1 *Reactivity of MAbs to denatured egg white RCP*

The effect of perturbation of the native structure of RCP by SDS, SDS+ßME treatment and reduction and carboxymethylation on its antigenicity towards the panel of MAbs was tested in ELISA. Figure 1 shows that most of the epitopes appear to be directed against conformation dependent epitopes and only 6B2C12 MAb is capable of interacting to an appreciable extent with the RCM-RCP. This is in accordance with the earlier results that the native 3D structure of RCP is required for its maximal antigenicity (Visweswariah *et al* 1987). The subtle differences in the binding patterns of various MAbs to denatured RCPs might be due to the different structural characteristics and/or susceptibility of the different epitopes to denaturation again emphasizing the individuality of these epitopes.

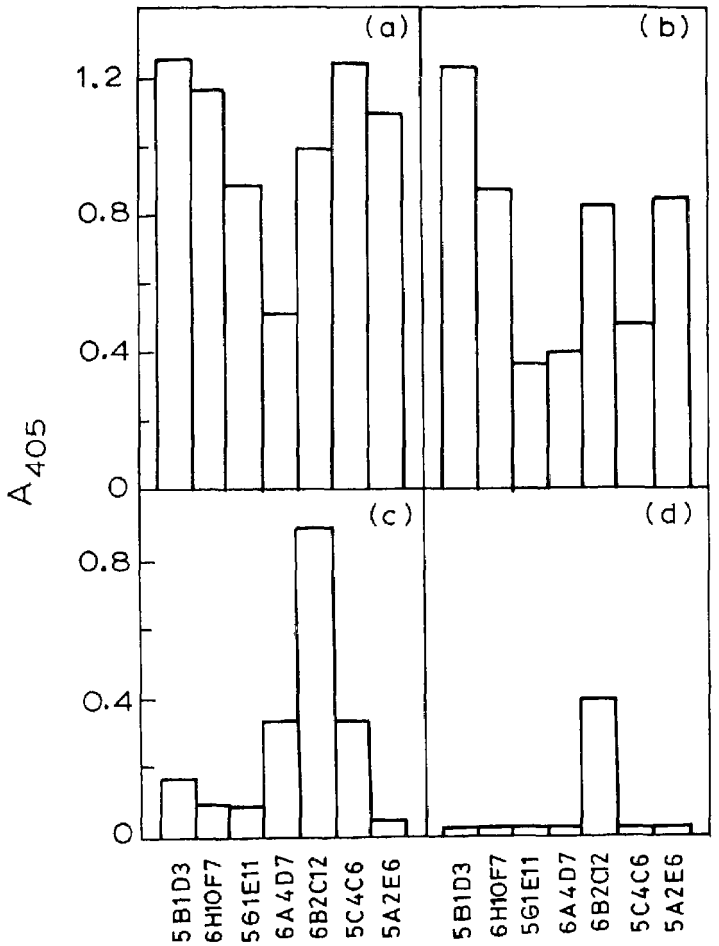

Figure 1. Immunoreactivity of the MAbs to denatured RCP.

Native egg white RCP (**a**) or egg white RCP denatured with either SDS (**b**), SDS + ßME (**c**) or reduced and carboxy-methylated **(d)** were coated at the concentration of 1 pg/well on polystyrene plates. Binding of the MAbs to the protein was determined in ELISA using anti-mouse IgG alkaline phosphatase conjugate.

3.2 *Binding of MAbs to egg white, yolk and plasma RCPs*

It is now well established that RCPs from chicken egg white and plasma are the products of the same structural gene and that plasma RCP is the precursor of the yolk RCP (White and Merrill 1988). During its yolk deposition, the plasma RCP is cleaved by specific proteolysis at the C-terminal end (White and Merrill I988). Thus yolk RCP is identical to the plasma RCP minus a 1I-13 amino acid C-terminal fragment and to the egg white RCP except for the constituents of the two oligosaccharide side chains attached to Asn^{36} and Asn147 residues and the cleaved C-terminal region. The MAbs were tested for binding to the three RCPs in order to determine whether any of them could differentiate these subtle structural differences between the three RCPs in LPRIA and SPRIA. Except for the 6B2C12 MAb (which did not interact with the yolk RCP but interacted with equal efficiency with both the egg white and plasma RCPs) (Karande *et al* 1991) all the other MAbs recognized all the three isoforms of chicken RCP. This finding implies that their corresponding epitopic conformation are determined primarily by specific folding of their peptidyl backbone and that the covalently bound carbohydrate moieties do not significantly contribute to MAb recognition. The binding region of 6B2C12 MAb has been mapped to be within the Gln^{203}–Glu^{219} residues (Karande *et al* 1991).

3.3 *Cross-reactivity of MAbs* with *RCPs from Different mammalian species*

We have earlier reported that mammalian RCPs have physicochemical properties similar to chicken egg RCP. Consequently RCP enriched fractions from mammalian pregnancy sera were used to investigate their binding abilities and specificities with a limited number of MAbs specific to chicken RCP (Visweswariah *et al* 1987). In order to extend these investigations to other MAbs, RCP enriched fractions from either rat, cow or human pregnancy sera were tested in heterologous LPRIA for displacement of [^{125}I]labelled egg white RCP bound to various individual MAbs. The concentrations (in ng) of the RCPs in the rat, cow and human sera (DEAE chromatography fractions) were derived from an inhibition assay employing polyclonal antibodies to chicken RCP. The standard chicken RCP concentration at 50% inhibition of the binding of the [^{125}I]chicken RCP to the antibodies was equated with the volumes of the RCP enriched fractions giving 50% inhibition. The concentration of the different RCPs in ng amounts capable of bringing about 50% displacement were calculated for each MAb. From the data of table 3 it is clear that all the epitopes specified by these MAbs are conserved in RCP through evolution of the mammalian species. Many of the MAbs appear to require lesser amount of the mammalian RCPs for 50% displacement of bound [^{125}I]labelled egg white RCP *vis-a-vis* chicken RCP. This may presumably be due to a lower estimate of the amounts of mammalian RCPs in LPRIA using the polyclonal antiserum, since the homologies among the various RCPs have not been calculated. On the assumption that these 7 MAbs represent the immune response of most, if not all, of the immunodominant, surface exposed epitopes on chicken RCP, the data in table 3 reinforce the concept regarding the high degree of evolutionary conservation of the overall epitopic conformations amongst different RCPs. The difference in the relative potencies of the various RCPs to bind to the MAbs are not

Table 3. Relative concentrations of chicken and mammalian RCPs required for 50% displacement of [^{125}I]labelled chicken RCP from conformation specific chicken RCP monoclonal antibodies.

Monoclonal antibody	Chicken RCP (ng)	Rat RCP (ng)	Cow RCP (ng)	Human RCP (ng)
5B1D3	1·4 (1)	1·0 (0·71)	1·8 (1·30)	1·5 (1·07)
6A4D7	5·0 (1)	2·5 (0·50)	3·5 (0·70)	2·9 (0·58)
5C4C6	3·6 (1)	3·0 (0·83)	5·0 (1·39)	6·7 (1·86)
6H10F7	27·0 (1)	11·0 (0·41)	6·8 (0·25)	16·0 (0·59)
5A2E6	34·0 (1)	32·6 (0·94)	5·4 (0·16)	67·0 (1·97)
5G1E11	70·0 (1)	58·0 (0·83)	ND	36·7 (0·52)

ND, Not determined.

Values in parentheses are ratios normalized with respect to chicken RCP, taking the concentration. of chicken RCP required for 50% displacement of [^{125}I]labelled RCP as unity.

ascribable to the difference in the Ka values of the MAbs is evident from the data of table 3. Though almost all the epitopes on RCP have been seen to be conserved from chicken to human, the immunodeterminants recognized by the various MAbs seem to have diverged to different extents during evolution. The epitope recognized by the MAb 6B2C12 was also seen to be present in rat and human RCP as determined by SPRIA (Karande *et al* 1991).

3.4 *Binding of MAbs to chemically modified chicken RCP*

Deglycosylation of RCP by HF did not appreciably alter the binding of the MAbs except that of MAb 6A4D7 which showed more than two-fold increase in the binding of the deglycosylated RCP in ELISA (table 4) suggesting that removal of the carbohydrate moieties by HF treatment gives a better accessibility for the antibody to bind to its epitope. This observation also suggests that the oligosaccharide side chains do not constitute a part of any of the epitopes recognized by the MAbs employed in this study. Dephosphorylation of the egg white RCP molecule while not affecting the binding of most of the antibodies, led to considerable (60%) decrease in the binding of the MAb 6B2C12 to the modified antigen. While the MAbs 5B1D3, 6H10F7, 5G1E11, 5C4C6, 6A4D7 and 5A2E6 showed an appreciably reduced binding to succinylated RCP, the MAb 6B2C12

Table 4. Immunoreactivities of chemically modified RCP as probed with monoclonal antibodies.

Monoclonal antibodies	Native RCP		Deglycosylated RCP		Dephosphorylated RCP		Succinylated RCP	
	Neat	1:10	Neat	1:10	Neat	1:10	Neat	1:10
5B1D3	2·60	1·75	2·5	2·4	2·50	1·30	1·04	0·72
6H10F7	1·08	0·80	1·2	1·0	0·85	0·80	0·65	0·45
6A4D7	0·96	0·70	1·8	1·7	1·00	0·70	0·42	0·31
5C4C6	0·88	0·60	0·9	0·6	0·83	0·45	0·39	0·26
5A2E6	0·78	0·70	1·0	1·0	0·50	0·50	0·36	0·32
5G1E11	0·85	0·70	1·3	1·0	0·65	0·65	0·35	0·29
6B2C12	1·35	1·20	1·9	1·5	0·55	0·50	0·05	0·05

Values represent absorbance at 405 nm in ELISA.

showed no binding to the same suggesting that either the lysine residues form an integral part of the antigenic determinants of chicken RCP or the intact lysine residues might help to maintain proper conformation of the epitopes (Visweswariah *et al* 1987; Ramanathan *et al* 1980).

3.5 *Immunoreactirity of the peptide from formic acid digest of the egg white RCP*

RCP digested with formic acid was resolved on SDS-PAGE. As can be seen in figure 2A, three peptide bands of molecular mass 25, 17 and 16 kDa were obtained

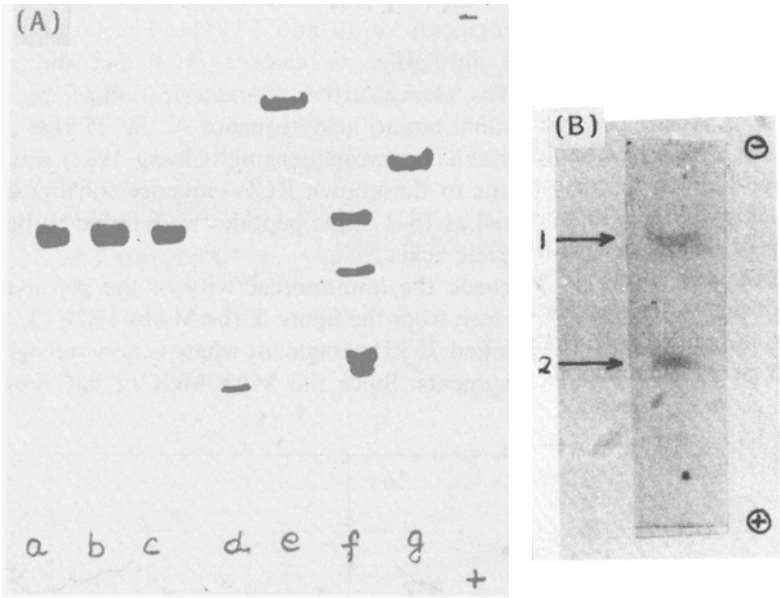

Figure 2. (A) SOS-PAGE pattern of formic acid digested egg white RCP.

50–75 m*g* of egg white RCP, digested with formic acid alone (a), formic acid in the presence of 7 M GnHCI and analysed under nonreducing condition (b), undigested RCP (c), digested with formic acid in the presence of 7 M GnHCI and analysed under reducing condition (f), were electrophoresed on a 8-25% gradient SDS-PAGE and stained with Coomassie blue R-250, α-lactalbumin (d), bovine serum albumin (e), ovalbumin (**g**) were run as markers.

(**B**) *In situ* phosphate staining of formic acid digest of RCP.

The formic acid digest of RCP electrophoresed on a 8-25% gradient SDS-polyacrylamide gel (as in lane 0 was stained *in situ* for phosphate with methyl green. 1→corresponds to uncleaved RCP (M_r, 37,000) and 2→to the 25 kDa fragment.

besides trace amounts of undigested RCP under reducing condition only. Under non-reducing condition i.e. in the absence of /3-ME, only one species with molecular mass corresponding to intact RCP was obtained indicating that the cleaved fragments are held together by disulphide bridges. In the absence of 7 M GuHCl, exposure to 75% HCOOH alone failed to cleave the Asp^{86}-Pro^{87} bond indicating that this chemical linkage is not accessible to cleavage by the acid in the absence of the denaturant. On *in situ* staining for covalently-bound phosphate on

polypeptides resolved in the gel, only the 25 kDa peptidyl fragment besides the undigested residual RCP (figure 2B) gave a positive reaction. It would appear that the 25 kDa fragment corresponds to the C-terminal region of RCP since both the egg white and the yolk RCP contain 8 phosphoserine residues exclusively confined to the peptidyl sequence 187-197 residues located towards the C-terminus (White and Merrill 1988). Similarly 16/17 kDa fragments resolving as a doublet on the gel apparently represent the N-terminal sequence l-86 residues of the RCP since they did not stain for bound phosphate. Based on the above argument, it is conceivable that the simultaneous liberation of 17 and 16 kDa N-terminal fragments during exposure to formic acid is attributable to partial deamidation of $Asti^{14}$ to Asp^{14} and subsequent cleavage of Asp^{14}-Pro^{15} bond resulting in the generation of two closely spaced polypeptide fragments of 16 and 17 kDa. The latter polypeptides reacted with the polyclonal antibodies to chicken RCP but did not exhibit reactivity with any of the MAbs. Hence further characterization of these fragments was not pursued. The N-terminal amino acid sequence of the 25 kDa polypeptide after four cycles of Chang's manual microsequencing (Chang 1983) was NH_2-Pro-Arg-Tyr-Thr which corresponds to the known RCP sequence starting from Pro^{87}. The yields of the 25 kDa as well as 16-17 kDa peptides were found to be 1% of the total protein digested with formic acid.

SPRIA was employed to check the immunoreactivity of the peptide fragments with all the MAbs. As can be seen from the figure 3, the MAbs 6B2C12, 5BlD3 and 5A2E6 interacted with the purified 25 kDa fragment whereas none recognised either the 16 or 17 kDa peptide fragments. Since the MAb 6B2C12 has been found to

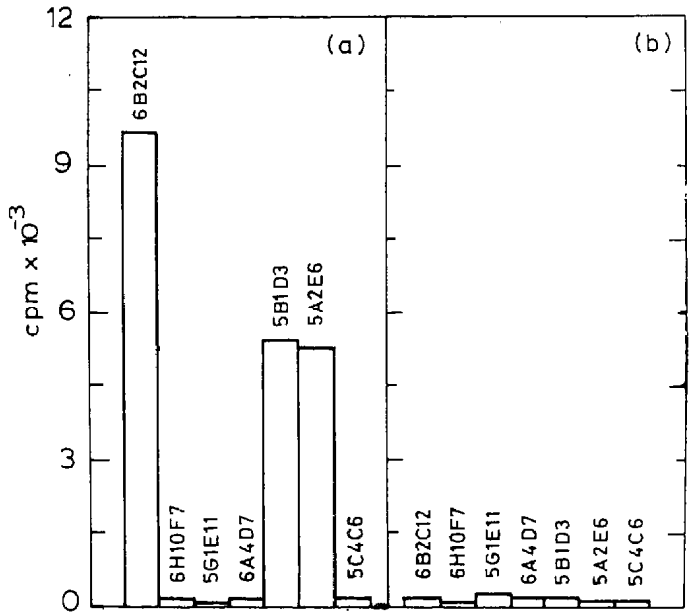

Figure 3. Binding assay of (a) 25 kDa and (b) mixture of 17 and 16 kDa polypeptides with monoclonal antibodies.

Either the 25 kDa polypeptide or a mixture of 17 and 16 kDa polypeptides (1 μg/well), purified and eluted from SDS-PAGE were coated on polystyrene plate. Binding of the MAbs to the peptides was determined by binding of [^{125}I]labelled protein A, through rabbit antibodies to mouse IgG.

recognize the extreme C-terminal tail-end fragment of chicken egg white RCP, it can be concluded that this 25 kDa fragment represents the entire sequence starting from Pro 87. The absence of interaction between the rest of the highly conformation dependent MAbs to any of the formic acid derived polypeptides clearly indicates that the conditions employed to generate and purify these fragments have resulted in abrogation of the native conformation of their epitopes. The above results also indicate that the assembled epitopes recognized by 5B1D3 and 5A2E6 MAbs have greater propensity to renature and hence to assume native-like conformation.

3.6 *Immunoreacticity of phosphopeptide*

The purified peptide exhibited a comparable mobility in 2D peptide map under similar conditions as reported earlier (White and Merrill 1988). The recovery of the peptide was about 2% of the total protein taken for digestion with trypsin. The two N-terminal amino acids were found to be NH_2-His-Leu which corresponds to the known RCP sequence starting from His'. The data presented in figure 4 indicate that the purified phosphopeptide harbours the antigenic determinant or a dominant part thereof recognized by the MAb 5B1D3, since a dose-dependent binding curve is obtained in SPRIA with this MAb. Surprisingly there was no discernible binding of this peptide to the MAb 5A2E6 despite the fact that in the native RCP this particular MAb recognizes a determinant overlapping with that of 5B1D3. It is therefore conceivable that 5A2E6 MAb recognizes a peptide region very close in space to, but not the phosphopeptide *per se,* yet is located within the amino acid sequence Pro^{87}-$G1u^{219}$ residues of RCP. This also may explain the partial inhibitory activity seen between 5B1D3 and 5A2E6 MAbs in the competitive solid phase inhibition assay.

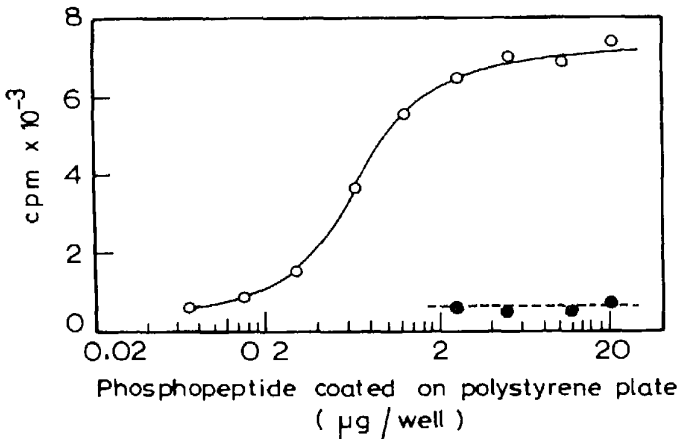

Figure 4. Binding assay of the phosphopeptide with monoclonal antibodies.

Varying concentrations of the isolated phosphopeptide (0·075–20 mg/well) were coated on polystyrene plate. The immunoreactivity of this peptide with the MAbs was determined by [^{125}I]labelled protein A binding through rabbit antibody to mouse IgG. (O), Binding curve of 5B1D3 MAb. (•), Binding curve of 6A4D7 MAb which was similar to all other MAbs.

4. Discussion

This paper describes the results of an extended study on the production and characterization of murine MAbs to chicken RCP and their use in studying the antigenic surface of RCP from different species. The epitope mapping studies indicated 7 unique epitopes (one epitope partially shared by two MAbs) on the surface of chicken egg white RCP molecule. Chicken egg yolk RCP which lacks C-terminal 11–13 amino acids bound only 6 among these MAbs. The assessment of the true number of a multitude of epitopes, usually depends on the size of the antibody repertoire and the available surface area on the antigen (Schwarz *et al* 1986) assuming that the surface of proteins is a continuum of antigenicity. Thus in the case of human chorionic gonadotropin (hCG) (molecular mass 38 kDa, Stoke's radius 30 Å and a glycoprotein) using 21 MAbs with which 9 epitopes were revealed, it has been surmized that the informational input was sufficient as to establish the number of epitopes present on hCG (Schwarz *et al* 1986). Similarly in the case of a related protein *viz.,* human follicle stimulating hormone, the use of a panel of 181 MAbs identified only 9 epitopes (Berger *et al* 1988). Thus the production and use of 18 MAbs to chicken RCP (molecular mass 36 K, Stoke's radius 25·5 Å and a glycoprotein) and the consequent identification of 7 surface exposed epitopes might represent, within reasonable limits, the majority of the immunodominant epitopes, exposed on its surface. However, it is important to point out that we failed to pick up any MAbs specific to a sequential epitope located within the N-terminus 16-17 kDa peptide of cRCP (figure 2A) obtained on formic acid digestion, eventhough this fragment was shown to react with the polyclonal antibodies raised to cRCP in mice (data not shown).

A stringent recognition between an MAb and its corresponding epitope has been emphasized by many investigators, since substitution of one amino acid in the epitope generally diminishes or abolishes its reactivity with its complementary MAb (Berzofsky *et al* 1982; Schroer *et a!* 1983). Even theoretical calculation based on the number of amino acids involved in an epitope has indicated that the possibility of finding two identical or even similar epitopes appear to be exceedingly small, unless the polypeptides displaying those epitopes are derived from a common ancestral gene (Marks *et al* 1985). The fact that almost all the epitopes are conserved across the species during evolution (table 3), again supports our earlier premise that the primary sequence of RCPs from different mammalian species is largely retained during evolution (Visweswariah *et al* 1987).

Recently the immunochemical analysis of the nature of the epitopes on many globular proteins have revealed that most of the determinants are of conformational type (Benjamin *et al* 1984) except that N- or C- terminii might give rise to sequential epitopes (Gnann *et al* 1989). In accordance with this, alteration of the tertiary structure of chicken RCP (SDS + βME treatment or reduction and carboxymethylation) resulted in the abrogation of the binding abilities of most of the MAbs except that of 6B2C12 MAb. Incidentally this MAb was found to interact with the C-terminus of egg white RCP (Karande *et al* 1991). It is noteworthy that chicken RCP undergoes extensive post-translational modifications like glycosylation at Asn^{36} and Asn^{147} (White and Merrill 1988) and phosphorylation of eight serine residues which occur as a cluster near the C-terminus of the vitamin carrier (White and Merrill 1988). Both of these covalent modifications have been

implicated in the receptor recognition sites for selective oocyte uptake and yolk deposition of RCP (White and Merrill 1988). Moreover a I1–13 amino acid peptidyl region at the C-terminus of RCP is removed by specific proteolysis as the protein crosses the oocyte membrane and/or is deposited in the yolk compartment. Thus it is conceivable that this peptide segment *per se* might be one of the receptor-binding site or is located in close proximity to it. In view of the above consideration the possibility of obtaining MAbs directed against the epitopes comprising these structural features was considered desirable.

Our search for MAbs recognizing two covalent modifications of chicken RCP implicated in its physiological functions (i.e., those binding to oligosaccharide and the phosphate residues *per se* which are involved in maximal oocyte uptake of the protein (White and Merrill 1988) has not been rewarding; however the MAb 5B1D3 was found to interact with the isolated phosphopeptide (His^{182}-Lys^{204}). It is relevant to point out that the phosphoserine residues may not be a part of the epitope recognized by 5B1D3 since dephosphorylation with acid phosphatase did not diminish the binding of the protein to this MAb. Though the binding of the MAb 6B2C12 to dephosphorylated RCP was found to decrease (table 4), that the phosphoserine do not form a part of its epitope is revealed by the observation that 6B2C12 recognizes the C-terminus 17 amino acid fragment of chicken egg white RCP (Karande *et al* 1991) which is away from the phosphoserine residues. Interestingly, the binding of the MAb 6A4D7 increased by nearly two-fold in ELISA once the antigen was deglycosylated (table 4) implying that the antibody might be recognizing an epitope which is partially masked by the carbohydrate moieties in the native form. These observations are supported by the studies of Skehel *et al* (1984) who have reported that the presence of oligosaccharide side chains can modify the antigenic structure of a haemagglutinin of influenza virus by effectively masking regions of the molecule that are antigenic in the variants that lack the glycosylation site.

The propensity of the MAbs directed against conformation dependent epitopes to recognize an isolated short stretch of peptidyl sequences present on the native antigen has been an intriguing observation in recent years (Bidart *et al* 1988). In the present study, we have clearly shown that MAb 5B1D3 interacted with the isolated phosphopeptide in a dose dependent manner. This contrasts with the data of figure 1, wherein RCM-RCP did not discernibly bind the MAb 5B1D3. The binding of the isolated phosphopeptide to the MAb might possibly be due to the greater freedom attained by the isolated short-stretch of peptides to assume different conformations, including those corresponding epitopes present on the native protein, so as to interact with a complementarily fitting antibody (Bidart *et al* 1988).Of relevance in this context is the finding that the Pro^{87}-Glu^{219}, C-terminal peptidyl sequence of egg white RCP obtained by formic acid digestion react with 5B1D3, 5A2E6 and 6B2C12 MAbs. This may be due to the greater propensity of these epitopes to renature and/or preferential preservation of these epitopes despite the peptide bond cleavage and the harsh experimental procedures used to isolate them. Supporting evidence for this premise stems from the failure of other MAbs to bind to either the N- or C-terminal fragments derived from formic acid cleavage of Asp-Pro bond. Obviously, the final proof regarding the exact locations of these antigenic determinants, their spatial orientation and the amino acid sequences contributing to their fine structures should await the elucidation of 3D protein folding pattern of

RCP by X-ray crystallographic studies (Berzofsky 1985) and testing the immunoreactivity of overlapping synthetic peptides spanning the entire primary structure of this protein (Geysen 1985).

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