A novel method for mapping assembled epitopes in batches: Identification of three epitopes at the receptor binding region of human chorionic gonadotropin

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Identification of conformation-specific epitopes of hCG β has been done using a simple batch method. Chemically or enzymatically-modified hCG β has been prepared in a batch and the effect of modifications on the integrity of different epitope regions has been investigated in a quantitative manner using monoclonal antibodies (MAbs) immobilized on plastic tubes from culture supernatants. Based on the extent of damage done to different regions by different modifications, three conformation-specific epitopes of hCG β have been identified. The method has been shown to have important advantages over the existing methods on many considerations. Using this approach, these epitopes have been shown to be at/near the receptor-binding region.

IDENTIFICATION of epitopic regions contributes to the understanding of the structure–function relationship of the protein antigen and has important relevance in the field of virology, protein structure and immunology[1–3]. Hybridoma technology has helped in providing a probe to epitopic regions and presently identification of epitopes are done using several methods, but most of the work is focussed on identification of sequence-specific epitopes using synthetic peptides and pepscan approaches utilizing ELISA methodology for screening the peptide sequences. However sequence-specific epitopes form a very small component of the total antibody produced in the humoral system, and majority of the antibodies are directed against the conformation specific epitopes[4–7]. Attempts at identifying conformation-specific epitopes are relatively very few and generally adopt a methodology which can focus on only one epitope at a time, and are very demanding in terms of the laboratory infrastructure, purity of the antigens, antibodies and are often time consuming[8–13]. Recently we reported on the identification of an α-specific epitope of hCG, utilizing a relatively simple method. This method had several advantages, one of the most important being its feasibility to identify the epitopic regions in batches[14]. In this paper we describe the development of that method to a batch approach for epitope analysis and identification of three epitopes of hCG β using monoclonal antibody probes.

Materials and methods

hCG and hCG β were laboratory preparations. Both showed good homogeneity in SDS-PAGE. Contamination of hCGα in the hCG β preparation was found to be less than 0.1% as determined using solid phase radioimmunoassay (SPRIA) with immobilised α-specific MAb 16. Iodination grade hCG (CR 127) and hLH were gifts from NHPP, Bethesda, USA. All other reagents were of analytical grade. $Na^{125}I$ was purchased from Amersham. The production and characterization of the MAbs used in this study were reported earlier[15]. All MAbs (B12, 18 and 20) are conformation-specific and react with both hCG αβ and β subunit. Iodination was carried out according to standard method[16]. Immobilisation of MAbs from culture fluid, SPRIA, chemical modifications were all done as already described[14,17]. Modification of hCG β was carried out in the same way as done for hCG, except 200 μg was used for each modification.

Dissociation of ^{125}IhCG–MAb complex

The complex was obtained by incubating ^{125}IhCG with immobilized MAb on plastic tube in a total volume of 0.6 ml overnight and discarding the unbound ^{125}IhCG. The tube was washed once with RIA buffer and incubated with 0.6 ml of RIA buffer for dissociation to occur. At different times of dissociation the supernatant was discarded and the radioactivity bound to the tube was counted in a LKB multigamma counter.

Epitope identification

The strategy used is essentially same as reported earlier[14]. Chemically or enzymatically modified hCG and hCG β derivatives were prepared in batches, and the effect on the binding ability of immobilized MAbs were

quantitatively assessed in SPRIA. Differential stability of the epitopes to different modifications were correlated with the sequence information to identify the epitopic regions through a selection and elimination process as already described[14].

Results

Effect of modification of different amino acid residues of hCG β on the integrity of three epitopes, namely 12, 18 and 20 is shown in Table 1. It is seen that modification of –COOH groups and digestion with trypsin results in large loss of activity in all MAb SPRIAs. Modification at lysine and tyrosine as well as chymotryptic digestion results in significant loss of activity of epitope 12. In contrast, modification of tyrosine results in total loss of activity of epitope 20. It is also seen that the cross-reactivity pattern of these MAbs to hLH is different, with MAb 18 showing 100% cross reaction in one extreme and MAb 20 exhibiting virtually no cross reactivity in the other end. Cross reactivity with the hCG β is nearly 100% in all cases indicating that the combination of the α subunit does not result in any marked changes in the above epitopes.

Results presented in Table 2 show similar loss of activities of different epitopes to modification, as well as enzyme digestion of hCG αβ dimer. It should be noted here that tryptic digestion of acetylated hCG which hydrolyses the peptide bonds adjacent to arginine residues destroys the integrity of all the epitopes. Likewise the modification of the arginine side chains through phenyl glyoxal also results in losses of activity to > 80%.

The importance of different residues in epitope integrity as obtained from Table 1 is presented in Table 3. In all cases arginine and –COOH groups appear to be very crucial for the epitope structure. The proximity of amino acids to various epitope regions is obtained through the extent of inactivation for chemical modification and enzyme digestion. Based on these data the epitope region of all the three epitopes is shown in Figure 1.

Dissociation of immobilized MAb–^{125}IhCG complex is shown (see Figure 4). Dissociation is more from the MAbs 18 and 20 (50% in 2 h) and less from MAb 12 (20%), and indicates higher affinity of MAb 12 to hCG, compared to the other two MAbs. In contrast to MAbs, PAb–^{125}IhCG dissociated to < 5%.

Table 1. Effect of chemical and enzymatic modification of hCG β on the immunoreactivity of different epitopes

Derivative	A.A. modified	Retention of activity (%)* in SPRIA using MAb 12	MAb 18	MAb 20
hCG β		100	100	100
TNBS	Amino (K)	39	100	100
TNM	Tyrosine (Y)	65	90	<1
CDI	COOH (D/E)	4	9	19
HCOOH		100	120	125
CNBr	Meth	100	95	100
Trypsin	K,R	<2	<1	<2
Chymotryp	Y.F.	70	90	100
CPAse		100	100	100
LAPase		100	100	100

*Activity measurement represents the average of two assays. Interassay and intraassay variation (14%)

Discussion

Identification of epitopic regions have been done primarily on the basis of chemical modification data (Table 1) of

Table 2. Effect of chemical and enzymatic modification of hCG on the immunoactivity of epitopes

Derivative	% activity* by MAb–SPRIA for epitopes 12	18	20
TNBS	20	90	100
TNM	35	108	35
CDI-DAE	20	26	55
Acetyl	100	100	100
Ac.hCG-Tryp	4	5	<2
Ac.hCG-Phe.Gly	10	14	<2
Trypsin	4	6	5
Chymotrypsin	57	90	97
CPase	100	100	—
hCG β	100	100	100
hLH	10	100	2

*The values presented are the average of 2 measurements.

Table 3. Involvement of amino acid residues in epitope structure

A.A. residue	Epitopes 12	18	20
K: Side chain**	+	–	–
Peptide bond**	–	–	–
R: Side chain	++	++	++
Peptide bond	++	++	++
Y: Side chain	+	–	++
Peptide bond	–	–	–
Disulphide	++	++	++
COOH side chain	++	++	+
M: Peptide bond	–	–	–
Expected a.a. of the epitope*			
(a) at core	R, D/E	R, D/E	Y, R
(b) proximal	Y, K	–	D/E
(c) distant	M, F	M, F, K, Y	M, F

*It is arbitrarily taken that if the retention of activity is <10%, the a.a. is at the core (++), 10–75% proximal (+), and >75% away (–) from the epitope.
**A loss of activity resulting from chemical modification is assigned to the side chain while the loss of activity resulting from enzymatic hydrolysis is assigned to the C-terminal peptide bond.

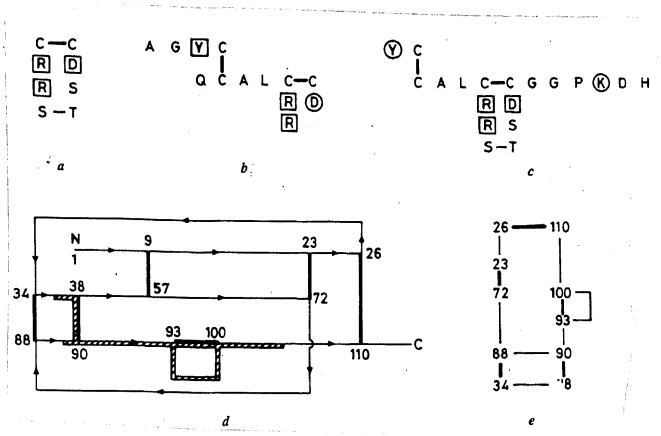

Figure 1. Schematic presentation (not drawn to scale) of the regions of the three epitopes identified by *a*, MAbs 18; *b*, MAb 20; and *c*, MAb 12; *d*, Combination of the three overlapping regions of the epitopes (hatched) drawn schematically in the hCG β molecule (not drawn to scale); *e*, Structural stabilization of epitope 12 is explained on the basis of the schematic representation of the epitopic region through disulphide bonds. Solid lines represent the disulphide bridges. Single letter codes of amino acids have been used. Amino acid residues identified to be at the core region of the epitope have been put in a square box, those at the proximity by circle.

hCG β and uses enzyme digestion data as an adjunct, as it is well documented that certain regions of hCG are not susceptible for tryptic digestion[18,19]. This observation is supported by our data[14] where we have seen that only 12 bonds are hydrolysed by trypsin (of the possible 18 as seen by sequence data) even at very high concentration of trypsin, and matches with data obtained by chemical approaches (13 susceptible bonds)[18–19] (Figure 5). Unlike in enzyme digestion where the sterical factors can prevent the approach of the active site of the enzyme and macromolecular substrate (in this case hCG), chemical modifications do not have such sterical problems, with most of the chemical modification reagents having easy access to the reacting amino acids, and hence our primary identification would be based on chemical modification data. For instance epitope 20 consists of an R&Y at the core of the epitope with a D/E in proximity. In addition this region cannot have a methionine or a chymotrypsin susceptible F/Y. All the above criteria are met by the region presented in Figure 1 *b*, 2 *b*. Enzymatic digestion data support this conclusion because the epitope though inactivated by TNM is not inactivated by chymotryptic digestion and hence the tyrosine identified should be resistant to chymotryptic attack. Likewise, the R residue/s should not only be close to Y but should be easily digested with trypsin. Infact in hCG there are only 4 digestible R residues at R43, R74, R95 and R104 (ref. 19). Of these R95 and R43 are close to Y37. Even though Y37 and R43 are in a sequence, this region has a methionine in proximity, and in addition does not have a D/E in proximity. Hence R43 as a part of the epitope 20 is eliminated. In contrast, Y37 is in proximity with R94/R95 through the disulphide bonds Cys38–Cys90, also has an aspartic acid at D99 brought close by Cys93–Cys100 and explains partial inactivation by CDI. R74 and R114 are digestible by trypsin, but do not have Y and D/E in proximity. Thus epitope 20 consists of amino acid residues Y37, R94, R95 and D99 brought closer by the disulphide bonds Cys38–Cys90 and Cys93–Cys100. Identification of the other two epitopes 18 and 12 are based on similar logic and is shown in Figure 1 *a*, 2 *a* and 1 *c*, 2 *c* respectively. The region of these over-lapping epitopes is presented diagrammatically in Figure 1 *d*. The rigidity imposed on this structure by interconnected disulphide bonds is obvious and hence explains the stability of these epitopes to several chemical and enzymatic modifications.

Such an approach is supported by the observation that epitope 12 shows marginal loss of activity on both tyrosine and lysine modification. It is seen that these residues are 9 residues apart in the nearest route. Considering that activity is lost (< 10% retention of activity) on modification of R and D, these residues are at the core region of the epitope. Residues K104 and Y37 are outside of the primary interacting/core region by as much as 4 residues and hence may have marginal role in the interaction with MAb, and hence partial loss of activity on modification. It is to be noticed that in the case of epitope 12, acetylation which provides a small extra group does not result in loss of activity (Table 2) whereas a larger hydrophobic group like TNBS results in a loss of 60% activity (Tables 1 and 2). Thus in chemical modification partial losses of activity may be a very useful index in identification of epitopes and may indicate the proximity of the specific amino acids to the primary epitope interacting/core region. It may be indicated here that modification of amino group at the core region of α-specific epitope resulted in greater than 90% loss in immunoreactivity[14]. In contrast, modification of K104 and Y37 proximal to epitope B12, showed partial inactivation even at very high concentration of the modifying agent (Table 1).

One of the observations which remains unexplained on the basis of the above identification method is the partial loss of activity of epitope 12 to chymotryptic digestion (30% – Tables 1 and 2). This loss is seen both in whole hCG as well as in its β-subunit digestion with chymotrypsin, and hence eliminates any role for the α subunit for this loss. The very marginal loss of activity can be easily explained on the basis of hydrolysis of Y82. When the cluster of the epitopes is expressed as shown in Figure 1 *e*, it is seen that epitope 12 has contributions coming from 34-38-90-105 sequence through disulphides, but the rigid loop structure is slightly disturbed by hydrolysis of Y82. This indicates that amino acid residues 86–87 may have a small marginal role in epitope integrity through sterical stabilization by the closed loop

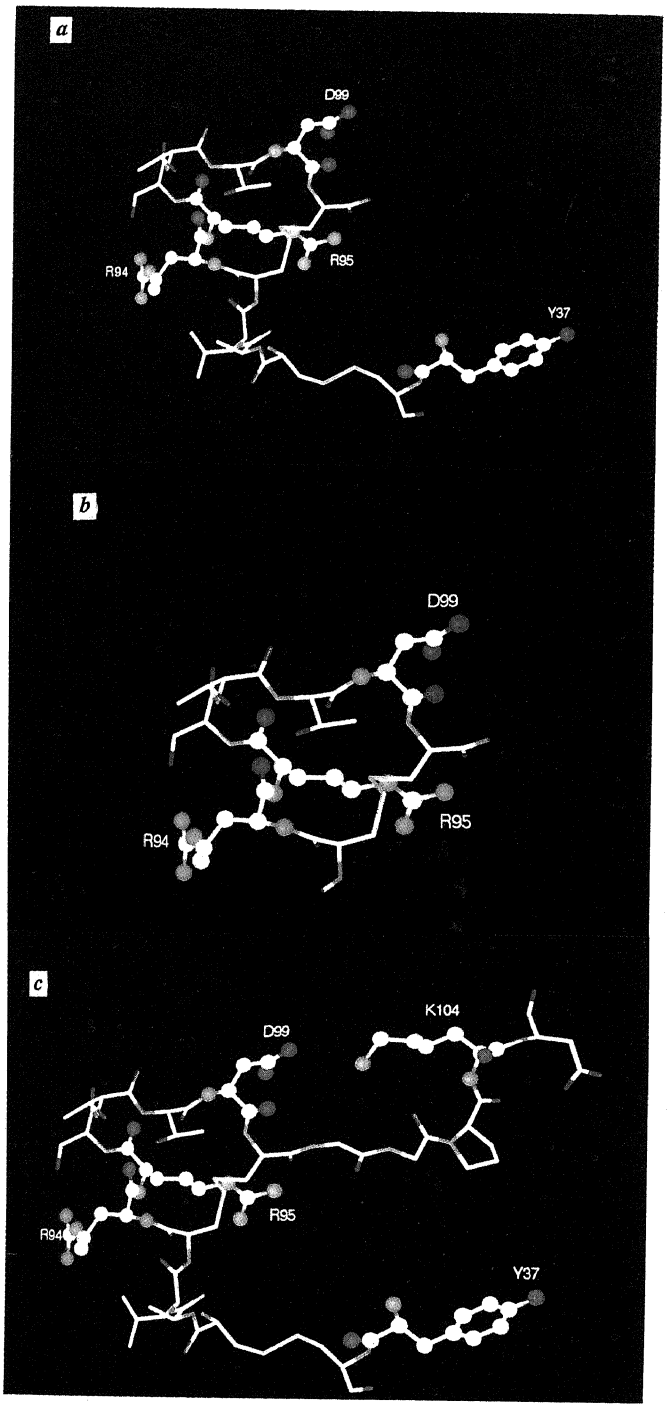

Figure 2. Stick model of epitopic regions of *a*, 20; *b*, 18; and *c*, 12 obtained through the Insight program using coordinates provided kindly by Isaacs. Side chains of amino acids identified by chemical modification approach are presented as balls and sticks. Disulphide linkages are coloured yellow. Atom colour-code: Red, oxygen; blue, nitrogen; white, carbon.

structure (26–88–90–110), or amino acid residues 86 and 87 may have a small contributions through their side chains for the epitope integrity. In contrast to this epitope, others do not have contributions coming from this region as chymotryptic digestion does not cause loss of activity of epitopes 18 and 20.

The cross-reactivity data on the hLH clearly substantiates the epitope identification. Epitope 18 shows almost 100% cross reactivity and if the sequence is seen, the loop 93–100 which is important for this epitope, has only one change in the sequence between hCG and hLH (T97 vs S97). In contrast, epitope 20 shows virtually no cross-reactivity and has three changes in Q89, A91 and L92 (replaced by R89, G91 and P92 in hLH). It is important to notice that a proline in the sequence can bring about significant change in the three-dimensional structure of the region. In contrast to these two, epitope 12 shows a partial crossreactivity of 10%, and has two changes at one end of its domain, (at A91 and L92) keeping the other 15 amino acids identical.

The pattern of loss of activity by chemical modification provides additional data about the size of the interacting region with its paratope. Thus, epitope 18 has a short size probably comprising of only peptide Cys93–Cys100, as modification of K104 and Y37 do not result in any activity changes. On the other hand epitope 12, though has R95 and D99 as its principle focus extends atleast up to Y37 on the N-terminal side and to K104 on the C-terminal side as their modification leads to partial loss of activity, and extends further at least to some extent to the region 72–88 of the sequence. Epitope 20 again appears to have its focus at Y37 and R95, and has the boundary on one side well within K104. However the boundary on the N-terminal side cannot be identified in this case as this region does not have amino acids which can be chemically modified. This region is not definitely stabilized by peptide region 72–88 as hydrolysis with chymotrypsin does not cause loss of activity. However, contributions of peptide chain 26–34 cannot be ruled out as this region does not contain any amino acid which can be modified by chemical/enzymatic means.

Pictorial presentation of the epitopes obtained by the analysis of the X-ray crystal coordinates of hCG (kindly provided by Isaacs) through the Insight program is presented in Figure 2 *a–c*. It is to be noted that each one of the amino acid residues that is identified is exposed to the solvent (Figure 3 *a*) and hence accessible to the antibody for combining. The composite epitopic region appears as a crevice in the ribbon model (highlighted in pink, Figure 3 *a*) as well as in the CPK model (highlighted in yellow, Figure 3 *b*). Amino acid residues identified by the above method are clustered in one region as seen by Figures 3 *a, b* and together form a region of access to the paratope either at the crevice seen clearly in Figure 3 *a* or a region, seen clearly in Figure 3 *b*, which can fit into a crevice of the paratope. Considering that the side chain residues are projecting towards the solvent, it is likely that the

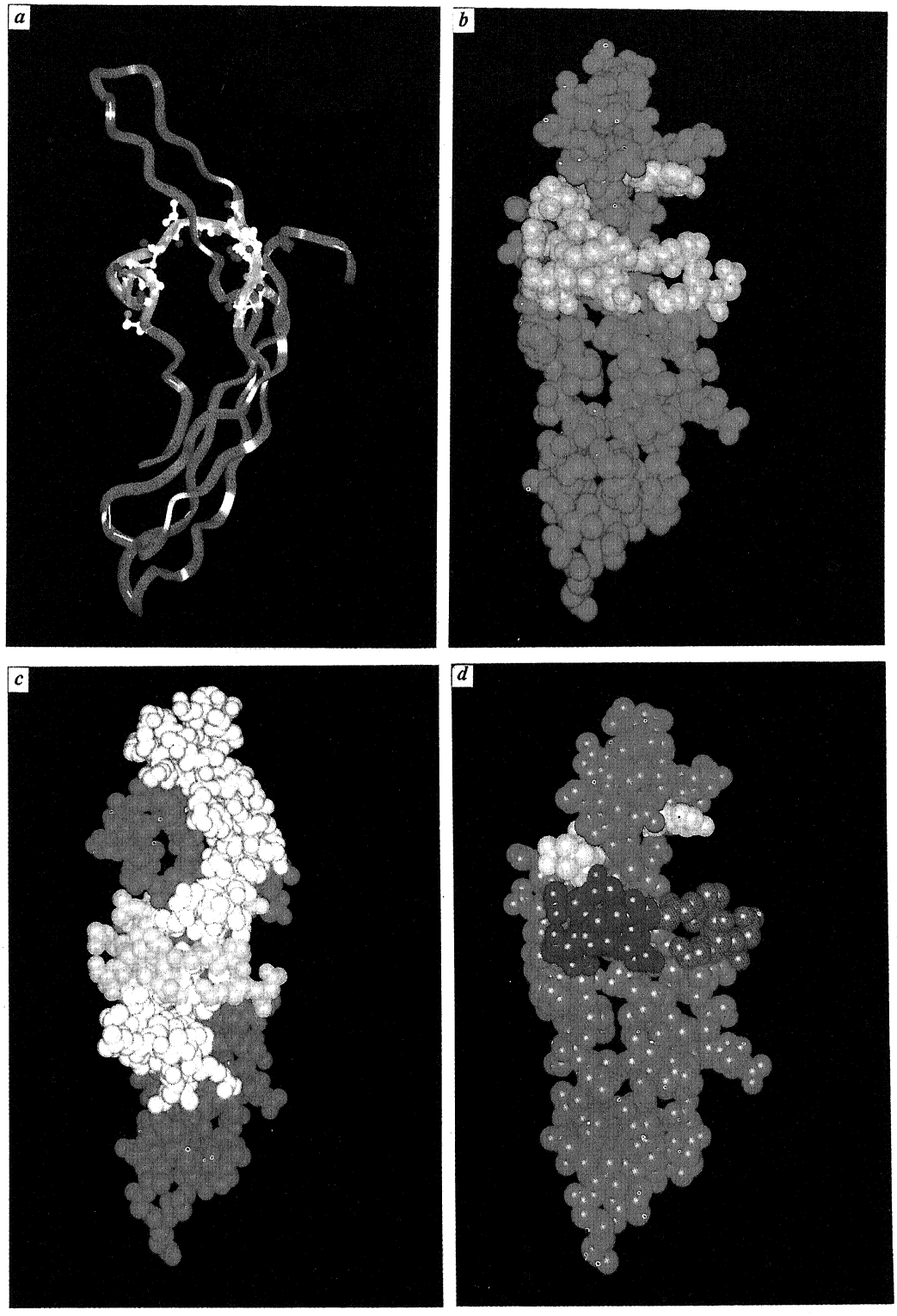

Figure 3. *a*, Ribbon diagram of hCG β highlighting the composite epitopic region (pink): Side chains of the amino acid residues identified by chemical modification are projected in ball and stick model. Atom colour code: same as in Figure 2; *b*, Space filling model of hCG β. Composite epitopic region is shown in yellow, rest of the subunit is coloured blue; *c*, Space-filling model of hCG αβ: Atoms of the α subunit are coloured pink and that of β subunit blue highlighting the composite epitopic region in yellow; *d*, Space filling model of hCG β: Regions of the composite epitope are colour coded as follows: Red, R93–D100; green, G101–D105; yellow, Y37-C38-C88-L92; The red region corresponds to epitope 18, red and yellow present the epitope 20 and red, yellow and green together represent epitope 12. Images are generated using Insight (Biosym) from the coordinates provided kindly by Isaacs[50].

region fits into a crevice of the paratope, than the paratope fitting into the crevice (Figure 3 *b*). In fact CPK model of the heterodimer (Figure 3 *c*) shows that α subunit (shown in pink) blocks the crevice implying the access of the epitope to the crevice of the paratope. Inactivation on chemical modification of side chains also indicates to the same conclusion.

Chemical and enzymatic modification data on whole hCG molecule (αβ dimer) very closely resembles those obtained with hCG β, indicating that the combination of α subunit with the β subunit do not change the integrity of these three epitopes.

Receptor inhibition data demonstrates that MAbs 18 and 12 fail to bind to receptor–hCG complex as well as inhibit the binding of the ^{125}IhCG to receptors implying that 12 and 18 are located at/or close to the receptor binding region (data not shown). However MAb 20 does not inhibit the binding of the hormone. Data presented in Figure 3 *a–d* in conjunction with the data on the epitope analysis (Table 3), indicate that the peptide loop Cys 93–Cys100 and the peptide region Cys100–His106 are in close proximity to the receptor-binding region and may form part of the receptor-binding region of hCG. Data obtained by chemical modification, use of synthetic peptides in competitive binding studies and site-directed mutagenesis[20–22] have indeed identified this region as receptor-binding region of hCG. Results obtained recently using chimeric constructs[23,24] have also unequivocally identified 94–114 as the receptor binding region. Thus, using this approach it is possible to identify the receptor-binding region of a hormone using MAbs as probes. These results also suggest that the region presented in Figure 1 *c*/2 *c* could be a viable synthetic substitute for obtaining receptor blocking antisera in either *in vitro* or *in vivo* systems, and also highlights the importance of the disulphide bridges in designing synthetic peptide substitutes for proteins in vaccine programmes.

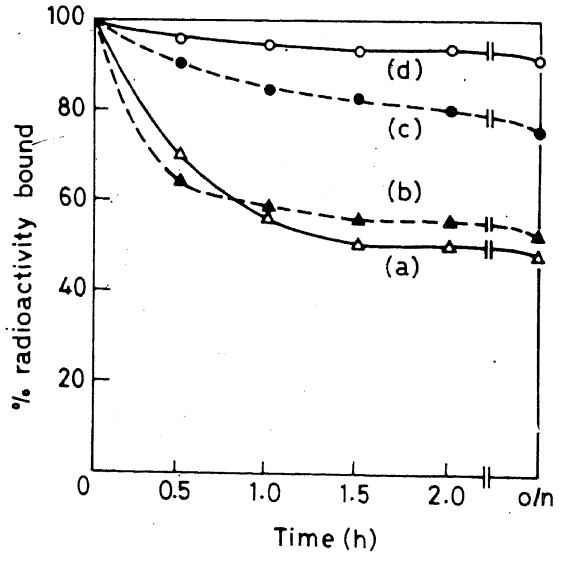

Figure 4. Kinetics of dissociation of the MAb–^{125}IhCG complex. Lines a, b, c and d represent the dissociation of the complexes of MAbs 20, 18, 12 and PAb (for hCG in sheep) respectively. Initial binding of ^{125}IhCG was 6k, 22k, 45k and 50 kcpm for (a), (b), (c) and (d) respectively.

MAb–^{125}IhCG complexes show significant dissociation of ^{125}IhCG from the tube. MAb 18 and 20 show higher dissociation both at 30 min (40%) and 120 min (55%). In contrast, the dissociation of the MAb 12–^{125}IhCG complex is only 10% at 30 min and 15% at 2 h. Based on the data provided in Figure 2 it is seen that the surface area of interaction of paratope with epitope 20 and 18 is about half of that of 12. Thus it is possible that the area of the epitopic region may have an inverse relation with the dissociation rate constant of the complex. Supporting this, MAbs 18 and 20 show a lower affinity constant compared to MAb 12. While the data provided above are not adequate to come to any conclusion, they definitely indicate the feasibility of understanding the epitope–paratope interaction in a rigorous quantitative manner using SPRIA probe coupled to modification studies.

Data provided so far have clearly shown that this method which utilizes the single step SPRIA as a quantitative tool for assessing the integrity of the epitopes on chemical/enzymatic modifications unequivocally identify the epitopic regions by simple and direct experimentation, through selection and elimination rather than isolation. Virtually all potential epitope-mapping strategies using overlapping peptides, Pepscan, combinatorial library, random peptide library depend essentially on the principle of peptide synthesis. They invariably compare the cross reactivity between peptide with protein antisera or protein with peptide antisera[25–27]. However the reactivity of the peptide depends on assay format (solution[28] or solid phase[29,30]), nature of the carrier protein chosen for conjugation, differences in the peptide accessibility and conformation, resulting in interpretational uncertainties[31]. In addition, heterospecific and nonspecific reactions are not uncommon[32,33]. The inability to consider the epitope in native context of the protein

01	S K^E P L R P R C R P I N A I L A V E K
21	E G C P V C I T V *N T T I C A G Y C P T
41	M T R^V L Q G V L P A L P Q V V C N Y R
61	D V R F E S I R L P G C P R^G V N P V V
81	S Y A V A L S C Q C A L C R R^S T S D C
101	G G P K^D H P L T C D D P R^F Q G S S S
121	*S K^A P P P *S L P S P *S R L P G P *S D T
141	P I L P Q

*Presents glycosylation sites, and ^ presents tryptic susceptible sites (ref. 17).

Figure 5. Primary sequence of subunit hCG β.

limits the possibility of extending these approaches to assembled epitopes, as evident from very few successful attempts[34–38].

Phage libraries displaying random peptides[39,40] are also of limited use[41] in mimicking discontiguous epitopes. Even the use of constrained peptide libraries is confined to identifying loop conformation[39] and mostly heterospecific sequences presumed as discontiguous epitopes due to the functional similarity[42]. Use of recombinant antigens, deletion mutants are often limited by the availability of suitable restriction sites, variation of expressibility of different regions and production of insoluble fusion proteins[43,44]. In addition the folding and antigenicity of the recombinant protein is questionable. These methods also need an expensive infrastructure with molecular biology expertise.

Approaches based on cross-species comparison using homologous protein, escape mutants and chimeric constructs have been employed for mapping epitopes[45,46]. However, availability of variants or mutants differing in the epitopic region limits the general applicability of this method.

Other chemical methods which have been used for identification of lysine residues at the epitopic region of cytochrome c by protection to acetylation provided by MAb[47], or protection provided by MAbs to tyrosine residues of TSH β for iodination[12] require pure antigen and antibody as prerequisites, and demands an infrastructure of microsequencing, HPLC, radiolabelling etc. and tends to be labour-intensive. In addition the sterical constraints are largely neglected.

X-ray crystallography of MAb–Ag complex is time-consuming and cumbersome, requiring a very large quantity of pure antigen as well as antibody for crystallization of each MAb–antigen complex and requires very specialized infrastructure[11,48,49]. Identification of each epitope is a major research task, and is likely to remain as an academic challenge, even though this is the only method presently available which recognizes the epitope in its native conformation.

All the methods described above, except X-ray crystallography and to a certain extent indirect chemical methods, are applicable only for sequence-specific epitopes. In peptide synthetic approaches, determination of the relative importance of amino acid residues is often assessed by ELISA methodology which suffers from several theoretical objections for quantification (Murthy *et al.*, unpublished data). These methods would also require expensive, elaborate and high tech infrastructure and are time-consuming. They identify a single epitope at a time. Above all, they are not amenable for batch method approach. In contrast, the method described in this paper is easy, relatively simple and least demanding on the infrastructure of the laboratory. In contrast to other approaches, this identifies the region by selection and elimination than by isolation/synthesis. A normal RIA set up is all that is required, and hence very easily adoptable in any laboratory. In addition this method identifies epitope in the native context of protein. To our knowledge this is the only method which identifies the amino acid residues of assembled epitopes using MAb culture supernatants in such low amounts (1–2 ml). While relative contributions of each of the residues in the epitope cannot be quantitated, it is possible in the case of hydrophilic residues which are amenable for modification, by adopting strategies of systematic quantitative approaches. Even in the above data the extent of contribution of K104 and Y37 to epitope 12 is obtained with no effort, a result almost impossible in any other existing method. This method by virtue of its batch approaches, uses the same modified derivatives in all quantitative assays and hence has the ability to identify the finer specificities of MAbs to very closely spaced or overlapping epitopes as is well demonstrated for epitopes 12, 18 and 20 in this paper.

The list of the probes by way of chemical modification and enzyme digestion used in the study is incomplete. Among group-specific modifications, amino groups, carboxyl groups and phenolic groups are modified. In chemical modification alone there are still modification reagents for His, Met, Trp etc. Likewise in enzyme digestion other specific proteases like V8 protease, and other proteases specific for various other amino acids have not been used. In spite of limited use of probes, the results of the study are valid. SPRIA can be further improved for a better quantitative assessment. Thus if more modifying reagents are used and precision of SPRIA is improved, this method of epitope analysis has the potential to assess quantitatively the role of atleast a few amino acids in the integrity of the epitopes, and will be in a position to locate the actual interacting regions of the epitope with the paratope, as has been shown for the epitopes 20, 18 and 12 of hCG β.

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ACKNOWLEDGEMENTS. We thank Dr Rajan Dighe for providing MAb culture supernatants and Prof. N. R. Moudgal for his continued encouragement. We also thank Prof. N. W. Isaacs, University of Glasgow, UK for providing the crystal structure coordinates for hCG. We also thank Mr Goutham Nadik for his help in use of Insight program. NV is a recepient of CSIR fellowship. Financial assistance from the Department of Biotechnology, New Delhi and Malladi Research Foundation, Madras is gratefully acknowledged.

Received 7 September 1995; revised accepted 21 November 1995