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Mapping the functional domains of human transcobalamin using monoclonal antibodies

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Recombinant human transcobalamin (TC) was probed with 17 monoclonal antibodies (mAbs), using surface plasmon resonance measurements. These experiments identified five distinct epitope clusters on the surface of holo-TC. Western blot analysis of the CNBr cleavage fragments of TC allowed us to distribute the epitopes between two regions, which spanned either the second quarter of the TC sequence GQLA…TAAM(103–198) or the C-terminal peptide LEPA…LVSW(316–427). Proteolytic fragments of TC and the synthetic peptides were used to further specify the epitope map and define the functional domains of TC. Only one antibody showed some interference with cobalamin (Cbl) binding to TC, and the corresponding epitope was situated at the C-terminal stretch TQAS…QLLR(372–399). We explored the receptor-blocking effect of several mAbs and heparin to identify TC domains essential for the interaction between holo-TC and the receptor. The receptor-related epitopes were located within the TC sequence GQLA…HHSV(103–159). The putative heparin-binding site corresponded to a positively charged segment KRSN…RTVR(207–227), which also seemed to be necessary for receptor binding. We conclude that conformational changes in TC upon Cbl binding are accompanied by the convergence of multiple domains, and only the assembled conformation of the protein (i.e. holo-TC) has high affinity for the receptor.

Vitamin B_{12} (cobalamin, Cbl) is absorbed in the distal ileum with the help of a specific binding protein intrinsic factor (IF) and appears in the circulation bound to another carrier transcobalamin (TC) [1]. Tissue uptake of the TC·Cbl complex (holo-TC) is mediated by specific receptors on the surface of the plasma membrane [2]. Holo-TC represents Cbl available for cellular uptake and a decrease in its level would indicate reduced absorption of the vitamin as well as systemic Cbl deficiency. Two new methods have recently been described for the measurement of holo-TC in plasma samples [3,4]. Both methods employ TC-specific antibodies to capture the protein from plasma but lack the specificity needed for direct measurement of holo-TC in serum. The antigenic determinants and the functional domains of TC have not been identified.

Cloning [5–7] and recent expression of several kindred Cbl-binding proteins [8–11] helped to elucidate some of their features. Thus, each of three human Cbl transporters (TC, IF and haptocorrin) consist of approximately 400 amino acid residues with 29–34%

Abbreviations
Cbl, cobalamin (vitamin B_{12}); ^{57}Cbl, [^{57}Co]cyano-Cbl; IF, intrinsic factor; RU, resonance unit; SPR, surface plasmon resonance; TC, transcobalamin; TC_p, recombinant human transcobalamin produced in a plant system; TC_{p11}, TC_{y31},…, the proteolytic fragments of TC_p and TC_y with the indicated molecular mass; TC_y, recombinant human transcobalamin produced in yeast.

pairwise identity between the mature proteins. Three conservative disulfide bridges are present in all members of this family according to the data for bovine TC [8]; however, only two central bridges seem to be important for Cbl binding and the stability of human TC [12]. A two-domain organization (289 + 110 residues) was suggested for a closely related protein IF [13]. Its small C-terminal domain could bind Cbl with unexpectedly high affinity [14] despite the absence of S-S bonds and the low number of conserved residues in this part of the sequence. Only later did the large N-terminal unit become involved in the binding of Cbl [13,14]. Assembly of two domains achieved the composite structure of the ligand-binding site and built the compatible interface between IF and its receptor [14]. The domain organization of TC remains unknown despite some progress in its crystallographic study [15].

We have described a number of TC monoclonal antibodies (mAbs) that interfere with the physiological functions of human TC, i.e. the Cbl and receptor binding [16]. Therefore, a map of the corresponding mAb-epitopes may reveal functional domains relevant for the biological activity of TC.

In this study we analyzed the binding of 17 TC-specific mAbs to the full-length protein and its fragments. This study identified regions that are likely to be involved in Cbl binding and interaction of holo-TC with the receptor on the cell surface.

Results

Epitope mapping using surface plasmon resonance

Epitope specificity was characterized using a set of 17 mAbs (Table 1), which were reacted with holo-TC pairwise. Three different protocols were used during the surface plasmon resonance (SPR) experiments (see Experimental procedures and Fig. 1). In each protocol, the interacting species were immobilized on the chip surface using a particular method, because the conjugation procedure often interferes with the 'true' binding results.

According to protocol 1, recombinant TC from yeast was immobilized on the chip via the first mAb attached to rabbit anti-(mouse epitope) IgG, whereupon the second mAb was injected (Fig. 1A). If the binding of the latter mAb was compromised, the epitopes of this pair were considered fully or partially overlapping (depending on degree of inhibition). Samples were combined in all possible permutations (Table 1). The data generated allowed us to define five distinct epitope clusters to which one or more of the mAbs bound.

Table 1. Binding properties of monoclonal anti-(human transcobalamin) IgG. All the data were collected according to SPR protocol 1.

mAb 1	K_D (nmol·L^{-1})	Subclass IgG	Epitope cluster	mAb overlapping with mAb 1
1-9	5–10	2a	3	5-18, TC4
1-12	5–10	2a	1	2-6, 3-5, 3-9, Q2-2, Q2-13
2-2	0.08	2a	2	3-11, 4-7, 5H2, TC7
2-6	< 1	1	1	1-12, 3-5, 3-9, Q2-2, Q2-13
3-5	1–5	1	1	1-12, 2-6, 3-9, Q2-2, Q2-13
3-9	0.04	1	1	1-12, 2-6, 3-5, Q2-2, Q2-13
3-11	0.08	2a	2	2-2, 4-7, 5H2, TC7
4-7	0.17	2b	2	2-2, 3-11, 5H2, TC7
5-18	10–100	2a	3	1-9, TC4
3C4	> 100	1	4	TC2
3C12	5–10	1	5	–
5H2	> 100	1	2	2-2, 3-11, 4-7, TC7
Q2-2[a]	5–10	–	1	3-9
Q2-12	5–10	2a	1	1-12, 2-6, 3-5, 3-9
TC2	1–5	1	4	3C4
TC4	5–10	1	3	1-9, 5-18
TC7	10–100	2a	2	2-2, 3-11, 4-7, 5H2

[a] Because of lack of the material, mAb Q2-2 could not be evaluated against all antibodies, however, the epitope specificity of this mAb is similar to 1-12 and Q2-12.

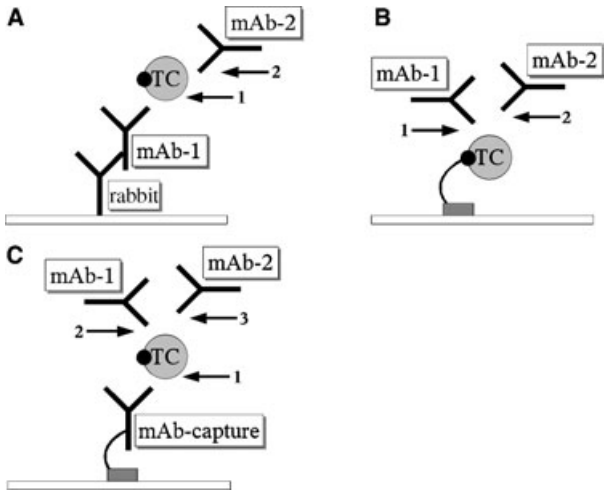

Fig. 1. Three different protocols of SPR binding experiments. Potential competition between mAb-1 and mAb-2 for the epitopes on the surface of holo-TC was investigated (see main text for details). (a) Protocol 1, (b) protocol 2, (c) protocol 3.

Nine representative mAbs, each reacting with one of the five clusters, were subjected to further SPR analysis using protocol 2 (Fig. 1B). In this setup, TC was immobilized on the chip as holo-TC via a Cbl analog, and two mAbs were sequentially injected into the

Table 2. Epitope specificity of the mAbs and the overlapping epitopes identified by SPR analysis. Values are expressed as % inhibition inflicted by the primary antibody bound to TC on the binding of a secondary antibody. The data were collected according to SPR protocol 2 except for those marked with an asterisk (*), which were obtained according to protocol 3 (see Experimental procedures). Bold indicates inhibition considered to be essential and reproducible.

Epitope cluster	First mAb	Antagonism of the binding to TC for second mAb (%)								
		3-9	4-7	5H2	2-2	TC7	TC4	3C4	TC2	3C12
1	3-9		23	32	10	26	0	0	0	27
				10*						5*
2	4-7	0	–	> **90**	**62**	> **90**	10	34	9	0
						> **90***		14*		
	5H2	7	**65**	–	**53**	**41**			1	0
							2	8		
	2-2	0	> **90**	> **90**	–	> **90**	22	35	2	**37**
								18*		**38***
	TC7	4	**80**	> **90**	**50**	–	22	18	4	15
3	TC4	0	18	36	20	35	–	4	2	26
				0*		3*				0*
4	3C4	0					0	–	> **90**	5
			27	23	17	13				
	TC2	0	19	31	11	5	0	> **90**	–	10
5	3C12	15	2	1	26	26	15	12	1	–

detection cell. The results on inhibition of the second mAb binding are presented in Table 2. The combined data from Tables 1 and 2 are in agreement with the scheme identifying five epitope clusters recognized by one or more of the mAbs.

In order to map the overlapping or neighboring epitopes, the additional SPR-binding protocol 3 was used (Fig. 1C). Thus, holo-TC was captured on the immobilized mAb TC2 because of its minimal antagonism with other antibodies (Table 2). Two other mAbs, likely to have the overlapping epitopes, were then sequentially reacted with TC. A relatively high competition of 90 and 38% was discovered only for the pairs 4-7/TC7 and 2-2/3C12 (Table 2). The capturing mAb TC2 could not be used to test 3C4-including sets because of their strong antagonism. Therefore, another TC capturing antibody 3-9 was used for pairs 4-7/3C4 and 2-2/3C4, where binding of the second mAb of the pair was inhibited by $< 20\%$.

Interaction of mAbs with the C-terminal domain of human TC was determined using peptides TC_{p11} and TC_{p12}, which originated from two adjacent cleavage sites. These 11–12 kDa fragments were isolated from the recombinant plants as a mixture of TC_{p12} (A_{320}ETIPQTQ…, 30%) and TC_{p11} (T_{326}QEIISVT…, 70%). Two proteolytic forms contained a considerable amount of bound Cbl according to high absorbance at 355 nm with the ratio of $A_{280}/A_{355} = 2.2$. The peptide-bound ligand did not dissociate during gel filtration or prolonged dialysis. Purified mAbs were immobilized on the CM5 chip, and the Cbl-containing fragments TC_{p11} + TC_{p12} (≈ 1 μM) were injected into the Biacore cell. The mAbs 3-9, Q2-2 (both epitope cluster 1) and TC4 (cluster 3) captured the above fragments with 126, 120, and 187 mRU of peptide bound per RU of antibody immobilized, whereas other mAbs did not. mAbs from two pairs, TC4 + 3-9 or TC4 + Q2-2, were able to bind to the same peptide simultaneously, whereas mAbs from the pair 3-9 + Q2-2 were not.

TC contains a binding site for the endogenous polysaccharide heparin [17]. The inhibitory effect of unfractionated heparin (12 kDa) on the interaction between holo-TC and various mAbs was tested. As shown in Table 3, the heparin-binding site overlapped with epitope cluster 5 and to some extent with cluster 4. However, low molecular mass heparin, used at the same USP units per mL, exhibited no corresponding inhibitory effect. This places the heparin-binding site of TC in the proximity of clusters 5 and 4, but without direct contact or overlapping.

Binding of TC_{p11} + TC_{p12} and human plasma TC to immobilized mAbs

Antibodies 3-9, Q2-2, 3-11, 4-7, TC4, 3C4, TC2 and 3C12 were immobilized on magnetic microspheres as described in Experimental Procedures. The mAb-containing microspheres were mixed with serum containing ^{57}Cbl-labeled TC in the presence or absence of 1 μM TC_{p11} + TC_{p12} [A_{320}ETIP… and TQEII… RLSW(326–427)]. The C-terminal peptides blocked the

Table 3. Specificity of monoclonal anti-(human transcobalamin) sera and their effect on the functional properties of transcobalamin. nd, not done.

mAb	Epitope cluster	Precipitation of Apo-TC (%)	Precipitation of Holo-TC (%)	Inhibition of precipitation by heparin[b] (%)	Blocking of recptor binding (%)	Blocking of Cbl binding (%)
3-9[a]	1	20–50	70–90	0–5	20–50	70–90
2-2[a]	2	70–90	20–50	nd	90–100	0–5
5H2	2	50–70	20–50	nd	90–100	0–5
TC7	2	70–90	50–70	0–5	90–100	0–5
TC4	3	70–90	50–70	0–5	50–70	0–5
3C4	4	50–70	0–5	50–70	20–50	0–5
TC2	4	90–100	50–70	0–5	50–70	0–5
3C12	5	50–70	50–70	70–90	90–100	0–5
Heparin[b]					70–90	0–5

[a] Data from earlier work [16]. [b] Data for unfractionated heparin at a concentration of 100 units·mL^{-1}.

binding of intact holo-TC to mAb Q2-2, 3-9 (epitope cluster 1) and TC4 (epitope cluster 3). However, they did not inhibit binding of holo-TC to mAb 4-7 (epitope cluster 2), 3C4 or TC2 (epitope cluster 4) and 3C12 (epitope cluster 5) suggesting that the corresponding epitopes are located outside the C-terminal region. Despite the fact that the above peptides compromised binding of TC·^{57}Cbl from plasma to mAb TC4, this effect did not increase during TC_{p11} + TC_{p12} saturation of the sample. Thus, even at a 10 000-fold excess of the peptides, mAb TC4 still captured 11% of maximal radioactivity, suggesting the matching epitope to be partially upstream of the sequence T_{326}QEII…

Effect of S-S reduction

All antibodies under study recognized recombinant human transcobalamin from yeast (TC_y) on western blot, if the protein were not reduced with dithiothrietol. Reduction of the disulfide bonds prior to electrophoresis abolished the binding of mAbs 3C4 and 5H2 (Fig. 2, see the corresponding lanes).

Binding of antibodies to CNBr peptides on western blot

Treatment of recombinant human TC_y with CNBr cleaved the protein after the 11 Met residues, and the peptides obtained were named after the corresponding cleavage sites (1–11). According to the nomenclature used, the elementary peptide 4 corresponded to the fragment between the fourth and the fifth Met residues [M↓GQLAL…DTAAM(102–198)]. As not all Met bonds in the TC sequence were cleaved completely, we obtained also a number of joined peptides, for instance, peptides 4–5 and 10–11, which comprised the sequences between Met residues 4–6 and 10–C-terminus. The mixture of the fragments was separated by HPLC, and the eluted peaks were analyzed by SDS/PAGE (Fig. 3). Each peak contained several TC_y peptides according to Coomassie Blue staining (upper panel). All bands were identified by N-terminal sequencing, and an analogous blot with the peptide fragments was incubated with a mAb. Two western blots (probed with mAbs 2-2 and 3-9) are shown in Fig. 2 (lower panels). Identical experiments were

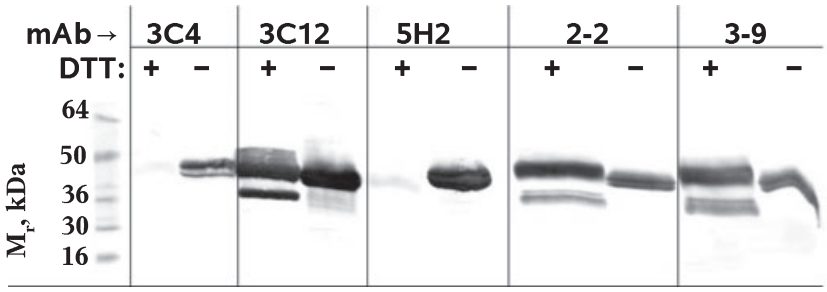

Fig. 2. Binding of anti-(human TC) sera to reduced and unreduced TC in a western blot. Recombinant human TC produced in yeast (TC_y) was subjected to SDS/PAGE with and without dithiothreitol reduction of the disulfide bonds followed by western blotting.

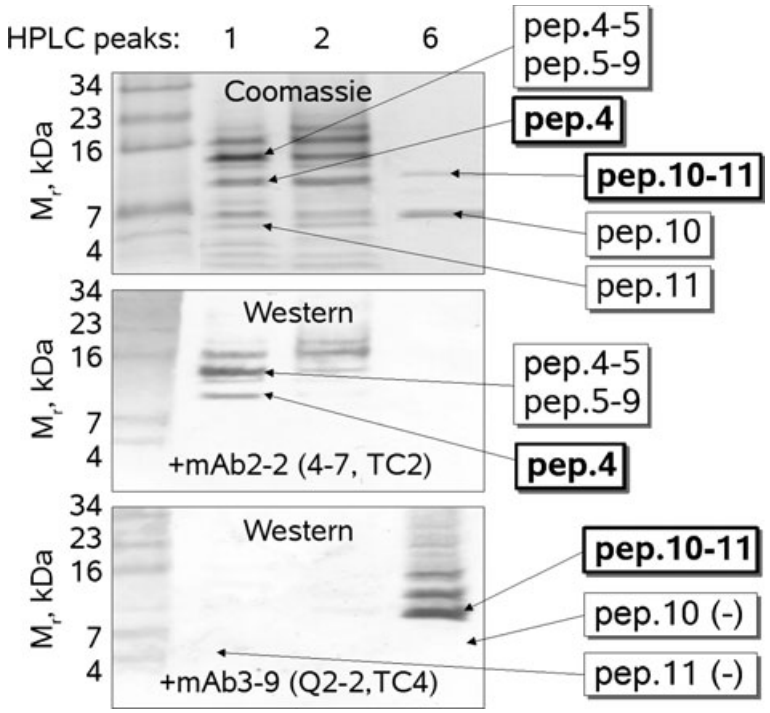

Fig. 3. Binding of the antibodies to CNBr cleavage peptides of TC_y. CNBr peptides of TC_y were fractionated by HPLC and subjected to SDS/PAGE and western blotting. (Upper) Blot stained with Coomassie Brilliant Blue. (Lower) Western blots of the same composition incubated with a TC-specific antibody. All peptides were identified by N-terminal sequencing, and several relevant fragments are indicated in the figure (see the main text). The smallest peptides with the antigenic properties are shown in bold type.

conducted with mAbs 4-7, 5H2, 3C4, 3C12, TC2, TC4 and Q2-2. The analyzed mAbs fell into three groups according to their binding patterns. Thus, the first group (mAbs 2-2, 4-7 and TC2) reacted with peptides, which contained the fragment 4 (Fig. 3, central panel). The second group (mAbs 3-9, TC4 and Q2-2) recognized the joined fragment 10–11, which remained only partially cleaved even after prolonged CNBr treatment (Fig. 3, lower panel). Neither of the latter mAbs attached to the separated peptides 10 and 11 as follows from the absence of the corresponding bands on the western blot. Antibodies from the third group (mAbs 5H2, 3C12 and 3C4) did not recognize any of the CNBr peptides. However, we can assign them to group 1 according to the map of overlapping epitopes presented in Tables 1 and 2.

Interaction of mAbs with the proteolytic fragments generated in the yeast expression system

The TC_y expressed in yeast resolved into three bands by SDS/PAGE. The major band corresponded to the full-length protein of 46 kDa (TC_{y46}), and two smaller ones originated from cleavage within the first quarter of the TC_y sequence according to the N-termini detected. The fragments were called TC_{y37} and TC_{y31} in accordance with their molecular masses on electrophoresis. All the above subforms of TC_y are likely to contain the native C-terminus as there was good correspondence between the theoretical and experimental molecular masses. Examination of the antibodies using western blotting (Fig. 4A, right) demonstrated identical patterns for mAbs from group 2 (the track for mAb3-9 is presented). These mAbs bound to all three major peptides TC_{y46}, TC_{y37} and TC_{y31}. However, among the group 1 mAbs, only 3C12 and TC2 bound to all the fragments, whereas 2-2 and 4-7 did not recognize TC_{y31} (Fig. 4A, lanes 2-2 and 4-7).

Interaction of mAbs with the proteolytic fragments generated in the plant expression system

The fragments of TC_p appeared from some endogenous protease activity. They had varying N-terminal ends, which were identified by sequencing (Fig. 4B). Based on molecular mass, all peptides contained the native C-terminus except for TC_{p28}, which had a molecular mass of 28 kDa (i.e. 5 kDa less than expected if the C-terminus were intact).

The pattern of immunoreactive bands by western blotting of TC_p was similar within the group 2 mAbs (3-9, Q2-2 and TC4, see the corresponding lanes in Fig. 4B). These mAbs reacted with the whole set of TC_p peptides. By contrast, mAbs from group 1 reacted only with certain fragments of higher molecular mass, 28 kDa or larger (Fig. 4B, 2,2 and 3C12, TC2, 4-7). Based on the alignment of peptide fragments and their reactivity with mAbs, epitope clusters 2, 4 and 5

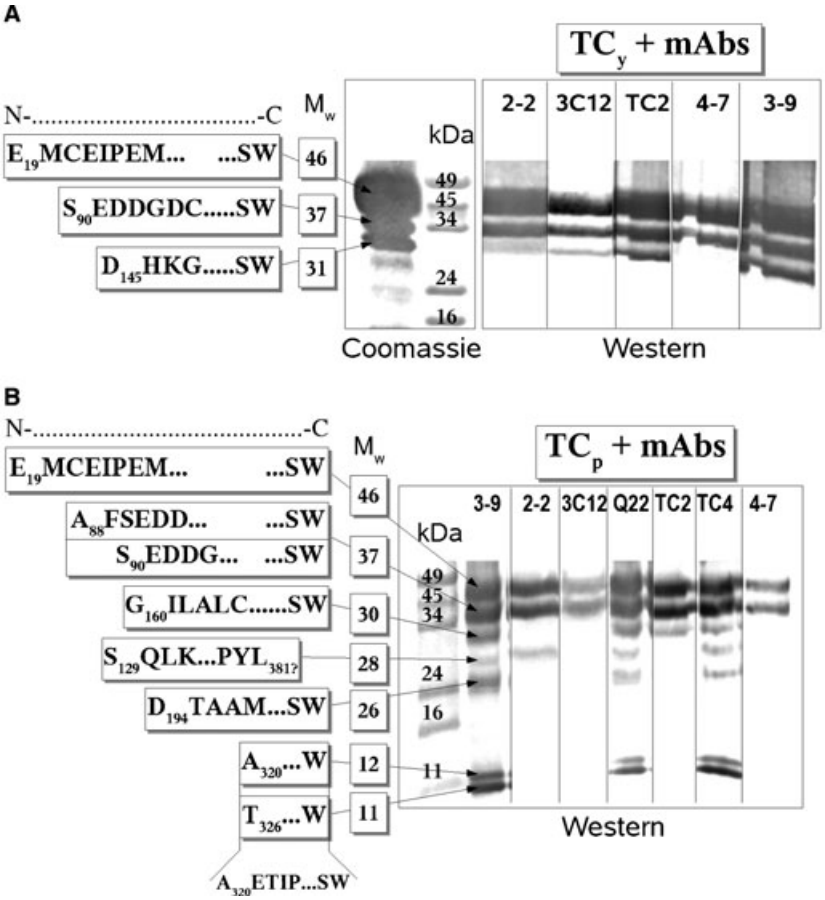

Fig. 4. Binding of antibodies to the proteolytic fragments of human TC from yeast and plants. (a) Fragments of TC_y. (Left) Coomassie Brilliant Blue-stained bands with the peptides identified by N-terminal sequencing. The left sketch depicts the fragments aligned and in accordance with their relative length. The theoretical molecular masses are shown in the small windows. (Right) Strips of the western blot after incubation with the corresponding antibody. (b) Fragments of TC_p (notation as in a). Strips of a blot were incubated with the indicated antibodies. The blots for mAbs 3-9, Q2-2 and TC4 reveal all the bands present on the electrophoresis according to Coomassie Brilliant Blue staining.

(group 1) were assigned to the second quarter of TC sequence, whereas epitope clusters 1 and 3 (group 2) were localized to the last quarter of the full-length sequence.

Antigenic properties of the synthetic peptides

Two synthetic peptides of 30 residues P_A and P_B were produced (see Experimental procedures). They imitated sequences of interest from the CNBr fragments 4 and 10–11, respectively. The synthetic peptides were tested for binding to mAbs 2-2, 3-9, 3C12, 4-7 and Q2-2, and the reaction was observed for two combinations (P_A + mAb 2-2) and (P_B + mAb 3-9). Three short peptides (c, d, e) from the region of the CNBr fragment 4 (adjacent to S-S bonds) failed to inhibit interaction between mAbs and full length TC (data not shown).

Interference of mAbs with the specific functions of TC

The effect of mAbs and heparin on Cbl binding and receptor recognition is shown in Table 3. Under the conditions tested, only one mAb, 3-9, in this set partially inhibited binding of Cbl (100 pM) to TC (50 pM). At the same time, the complex mAb3-9·TC could be saturated with Cbl at higher concentrations (1–10 μM) according to SPR data and spectral measurements. The specific absorbance shift of TC·Cbl [9] was also reproduced for the mAb3-9·TC·Cbl complex. In other words, the final organization of the Cbl binding site of TC seemed to be restored disregarding the attached antibody when sufficiently high concentration of Cbl was used.

A number of mAbs suppressed interaction of the mAb·TC·Cbl complex with the specific receptor (Table 3). Unfractionated heparin (100 $U \cdot mL^{-1}$) also noticeably inhibited binding of holo-TC to the receptor, but not to Cbl (Table 3). In addition, unfractionated heparin (but not low molecular mass heparin) inhibited the binding of two mAbs to holo-TC at $IC_{50} = 18$ and 310 $\mu g \cdot mL^{-1}$ for mAbs 3C12 and 3C4, respectively. It would appear from the above data that the positively charged heparin-binding region is in the proximity of epitope cluster 5 and is involved in the holo-TC–receptor interaction.

Discussion

Based on the patterns of mAb binding to native TC (Fig. 1, Tables 1 and 2) and the CNBr peptides (Fig. 3) the antibodies fell into two groups that could be further divided into five subgroups (epitope clusters). Group 1 (mAbs 4-7, 2-2 and TC2) recognized CNBr peptide 4, GQLA…TAAM(103–198) (Fig. 5A, red solid underline), which localized epitope clusters 2 and 4 within this sequence in accordance with Tables 1 and 2. Several related mAbs (5H2, 3C12 and 3C4) did not interact with the blotted peptide 4, however, their binding to the native TC was competitive with antibodies of group 1, see Tables 1 and 2. This places all the corresponding epitope clusters (i.e. 2, 4, 5) inside the sequence of the fragment 4 (Fig. 5A).

The second group of antibodies (TC4, Q2-2 and 3-9) bound uniformly to the uncleaved CNBr peptide 10–11, LEPA…LVSW(316–427) (Fig. 5A). Interestingly, none of the mAbs recognized the two separated fragments of this peptide, LEPA…TSVM(316–385) and GKAA…LVSW(386–427) (Fig. 3, lower panel, respectively 10 and 11)). Absence of interaction in this case could be due to either loss of the epitope or an artifact of the blotting procedure. The location of peptide 10–11 along the primary structure of TC is shown in Fig. 4A as sequence with a blue solid underline.

The natural proteolytic cleavage of the recombinant TC during expression of the protein in yeast and plants provided an opportunity to examine the antigenic properties of these peptides. The results of western blotting showed that step-by-step shortening of the original TC sequence was accompanied by loss of the immunological reaction with the antibodies (Fig. 4). Correspondence between a truncated sequence and loss of the binding to a specific antibody identified several smaller segments inside the long peptides 4 and 10–11 that comprised the relevant epitopes. The results of analysis are presented in Fig. 5A, where the epitope clusters are shown in different color.

The positions of the epitopes for mAbs 2-2 and 3-9 were further defined with the help of two synthetic peptides, P_A and P_B (red and blue dashed lines, respectively, in Fig. 5A), which localized the epitope for mAb 2-2 in the sequence GDRL…HPHT(124–152) and that for mAb 3-9 within TQAS…QLLR(372–399). None of the other antibodies recognized the above peptides. The smaller synthetic fragments (c, d, e) imitated other segments of the CNBr peptide 4 in Fig. 4A, but did not inhibit mAb binding to the intact protein. The lack of competition in the latter case could not be interpreted unequivocally because the small size of these peptides may not adequately cover an epitope.

Antibodies 5H2 and 3C4 did not recognize TC with reduced disulfide bridges (Fig. 2). However, these mAbs bound to the native protein with intact S-S bonds and this binding was competitive with the well-characterized antibodies 4-7 (epitope cluster 2) and TC2 (cluster 4), respectively (Tables 1 and 2). This places the S-S-dependent antigenic sites in the vicinity of Cys residues of the orange and green segments in the sequence (Fig. 5A). In this figure we present the scheme of the S-S bonds for human TC based on our previous data for bovine TC [8]. As we did not detect any free Cys residues in human TC [9], we presume cysteines C83 and C96 are connected, which contradicts the results of Kalra *et al.* [12]. The disulfide sensitive antibodies 5H2 and 3C4 may be conformation specific. In this case, the lack of binding to the reduced TC is caused rather by loss of the correct three-dimensional organization than by disruption of the S-S bond, per se.

The effect of heparin on interaction between mAbs and TC was evaluated because the positively charged sequence KRSN…RTVR(207–227) (a potential heparin-binding site) was in a neighboring position to epitope clusters 4 and 5. The inhibition of mAbs binding by unfractionated heparin (but not low molecular mass heparin) confirmed the heparin-binding site (Fig. 5A, cyan sequence) to be in the proximity of, but not overlapping with, clusters 4 and 5.

In order to visualize the epitopes identified by mAbs within the native structure of TC, a computer-based three-dimensional model of apo-TC was produced on the basis of its primary structure (see Experimental procedures section). The accuracy of the model cannot be validated because of the lack of any homologous structures, and Fig. 5B is used for visualization purposes. The epitope clusters and heparin-binding site are somewhat dissipated along the sequence. However, it is known that TC and IF change their conformations upon attachment of Cbl, which results in reduced Stokes radius [18]. In addition, recent data have shown that Cbl assembles distant domains of IF in a more compact structure with high affinity to the ligand and the specific receptor [13,14]. One can hypothesize the same transformation for the kindred protein TC.

The blocking effect of some mAbs on the functional properties of TC (this study, [16]) supplemented the epitope mapping and provided a deeper insight into operation of the TC domains. The binding of TC·Cbl to the receptor was suppressed by many antibodies, as well as by heparin (Table 3). Only an effect of 70% was considered to be specific, which narrowed the set

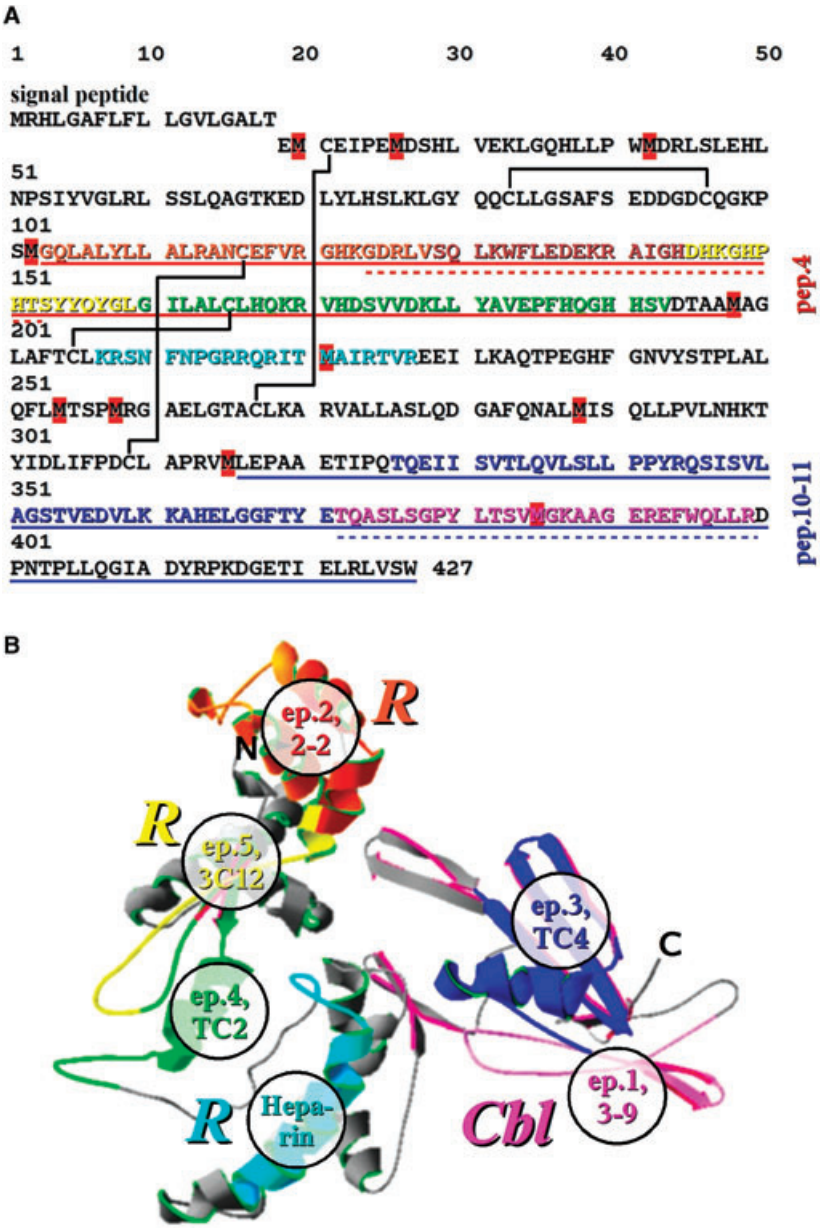

Fig. 5. Location of the epitopes identified along the primary amino acid sequence and in the simulated three-dimensional model of transcobalamin. (a) The sequence of human TC is presented with the putative epitopes and the heparin-binding region indicated in different colors: epitope cluster 1 (violet); epitope cluster 2 (orange for mAb 4-7, brown for mAb 2-2); epitope cluster 3 (blue); epitope cluster 4 (green); epitope cluster 5 (yellow); heparin-binding site (cyan). Positions of the peptides are underlined as follows: CNBr peptide 4, red solid line, GM(103–198); CNBr peptide 10–11, blue solid line, LW(316–427); synthetic peptide A, red dashed line, GH(124–151); synthetic peptide B, blue dashed line, TR(372–399). Disulfide bonds are indicated with black lines. Methionine residues of mature TC are highlighted with red. (b) Computer-simulated three-dimensional model of transcobalamin. The N- and C-termini are indicated by the corresponding letters (N-terminus is hidden behind the α helix). The colors of different regions correspond to the epitope clusters shown in (a). The letters R and Cbl indicate the suggested regions involved in the receptor and Cbl binding, respectively. They are deduced in accordance with the maximal mAb/heparin effect on the functional activity of TC. Arrows show the hypothetical movement of the domains after attachment of Cbl, see the main text.

of the receptor related domains to the epitope clusters 2, 5 [GQLA…QYGL(103–159)] and the heparin binding site [KRSN…RTVR(207–227)] (Fig. 5). As these sequences still represent a significant part of TC, a composite organization of the receptor recognition site may be suggested, where its components come from different parts of the protein. Reconstruction of the functional receptor-binding region requires convergence of several domains which can be accomplished only after attachment of Cbl to TC. The above scheme would explain the 28-fold higher affinity of holo-TC for the receptor when compared with apo-TC [2]. Composite organization of the corresponding site was also suggested for closely related protein IF [13,14]. In this regard, an earlier attempt to confine the receptor specific site of IF to a short sequence [19] does not seem to be quite justified.

In contrast to a considerable effect of multiple mAbs on the interaction between TC and the receptor, only mAb 3-9 caused noticeable suppression of Cbl binding to TC (Table 3). However, this mAb also bound holo-TC and could not preclude saturation of TC with Cbl when the reactants were taken at higher concentrations. The latter suggest that the epitope containing region (Fig. 5, magenta segment) is not directly involved in Cbl binding but likely resides in the pro-

ximity of the Cbl binding site. Sufficiently strong retention of Cbl by the isolated C-terminal peptides TC_{p11} and TC_{p12} and the analogous data for the C-terminal fragment of intrinsic factor [14] supports this conclusion. The other antibodies in this assay did not hinder the interaction of TC with Cbl at all (Table 3) and therefore we cannot draw any conclusions on the involvement of the other parts of TC in Cbl binding. However, we do not think that ligand binding occurs exclusively at the C-terminus of TC. Conjugation of a Cbl derivative to TC [10], analysis of alignments for several Cbl-transporting proteins [6,8,20] and the complex character of the Cbl-binding kinetics [9] point to multiple contacts between the ligand and the specific protein. Accordingly, the C-terminus of TC is likely to be a critical but not sufficient element in the Cbl-binding process. This was clearly demonstrated for IF, when the ligand induced assembly of the split N- and C-terminal fragments of this protein [13,14].

In conclusion, we have identified epitopes for several mAbs derived against the human cobalamin-binding protein TC. This mapping has provided valuable information on the organization of the Cbl and receptor binding sites of TC. As a consequence, one is better able to understand the specificity of this protein for Cbl, the physiological significance of holo-TC and ultimately how this protein interacts with the cell surface receptor to mediate the cellular uptake of Cbl.

Experimental procedures

Materials

All salts and media components were purchased from Merck (Darmstadt, Germany), Roche Molecular Biochemicals (Mannheim, Germany), Sigma-Aldrich (St Louis, MO, USA), Becton-Dickinson (Sparks, MD, USA). Encapsulated magnetic microspheres (EM1100/40; mean diameter, 0.86 μm) coated covalently with goat anti-(mouse IgG (H + L)) Ig were from Merck-Eurolab SAS. ^{57}Co-labeled Cbl was from ICN Pharmaceuticals Ltd. (Basingstoke, UK). Unlabeled Cbl, unfractionated heparin and low molecular mass heparin from porcine intestinal mucosa were from Sigma. Rabbit anti-(mouse Fc-γ) used for immobilization of murine mAbs on the BIAcore chip was from Biosensor AB (Uppsala, Sweden).

Proteins and antibodies

Expression and purification of recombinant human TC from yeast was performed as described elsewhere [9].

Expression and purification of TC from the recombinant plant *Arabidopsis thaliana* was performed identically to the procedure developed for a kindred cobalamin-binding protein intrinsic factor [11]. The last purification step was gel filtration, which separated the full-length TC_p (43 kDa) from its two C-terminal peptides TC_{p12} (12 kDa) and and TC_{p11} (11 kDa).

The production of human TC mAbs in mouse has been described previously [16].

SPR studies

SPR binding was performed using a BIAcore instrument (BIAcore Biosensor AB) according to the recommendations of the manufacturer.

Protocol 1

Rabbit anti-(mouse Fc-γ) IgG (30 $mg \cdot L^{-1}$) was immobilized on the surface of the carbodiimide-activated chip. The reaction was performed in 50 mM acetate buffer, pH 5.0 at flow rate of 5 $\mu L \cdot min^{-1}$, until the SPR signal reached ≈2000 RU over baseline. Unreacted groups were blocked using 1 M ethanolamine, and the primary antibody [mouse anti-(human TC) Ig, 10 $mg \cdot L^{-1}$] was captured on the chip in Hepes-buffered saline, pH 7.3, 3.4 mM EDTA, 50 $mg \cdot L^{-1}$ BIAcore surfactant. Mouse serum (1 : 10 dilution) was then injected in order to saturate the excessive binding sites on the anti-(mouse epitope) IgG chip.

Interaction of recombinant holo-TC with the antibodies on the sensor was evaluated at TC = 1 nM −10 μM, flow 5 $\mu L \cdot min^{-1}$. The chip surface was regenerated by washing with 10 mM HCl after each analysis The covalently immobilized rabbit anti-(mouse Fc-γ) IgG was stable and there was no significant decrease in the ligand binding during repeated washing and reuse of the chip. Data points were collected, and the rate constants for association and dissociation (k_{on} and k_{off}) were calculated. The equilibrium dissociation constant corresponded to $K_d = k_{off}/k_{on}$.

Protocol 2

Biotin–cobalamin (100 μM) was immobilized on the SA-chip via biotin-specific antibodies. The immobilized Cbl was saturated with apo-TC (1 μM) and two or more TC mAbs were consecutively injected. Suppression of the secondary mAb binding was evaluated. The proteins were stripped from the SA chip with 0.2 M glycine, pH 2.2 prior to reuse.

Protocol 3

Antibodies TC2 and 3-9 were biotinylated and bound to the streptavidin-coated Biacore chip. Holo-TC was then injected and immobilized on the chip via the capturing mAbs. Two more mAbs were sequentially injected, whereupon interference between the two latter antibodies was

estimated. To minimize antagonism between the capturing antibody and the mAbs under assay the following combinations were used: (a) capturing mAb TC2 plus pairs 3-9/5H2, 3-9/3C12, 4-7/TC7, 2-2/3C12, TC4/5H2, TC4/TC7, TC4/3C12; and (b) capturing mAb 3-9 plus pairs 4-7/3C4, 2-2/3C4.

Synthesis of biotinylated Cbl

Cbl was biotinylated at the ribose 5′-O position as described previously [21]. In short, Cbl was succinated at the ribose 5′ position, activated by EDC/sulfo-NHS, and conjugated with 1,12-diaminododecane. Finally, the Cbl derivative with the 12-carbon linker was conjugated to biotin using sulfo-NHS activated LC-biotin. The final product was purified by RP–HPLC and freeze-dried. Before use, the biotinylated Cbl was dissolved in methanol to 0.5 mM and then diluted with the appropriate buffer to the desired concentration (usually 10 μM in HBS-EP buffer).

Binding of [^{57}Co]Cbl TC to anti-(human TC) IgG in the presence of TC fragments

Monoclonal anti-TC IgG were bound to polyclonal goat anti-(mouse epitope) IgG that were covalently linked to magnetic microspheres as described previously [4]. TC in 1.8 mL of human serum was labeled with the radioactive ligand (300 pM of ^{57}Cbl, 30 min). For the experimental sample, 850 μL of the radiolabeled serum was mixed with 20 μL of TC fragment (TC_{p11} plus TC_{p12}, 1 μM final concentration). An identical aliquot of the serum mixed with 20 μL of the buffer served as the control. A 90 μL aliquot from each sample was incubated with 10 μL of the antibody-coated microspheres at room temperature for 1 h, the microspheres and supernatant were separated using a magnet, and the radioactivity in each fraction was determined.

Binding of mAbs to peptide fragments generated by CNBr treatment

Recombinant human TC from yeast was treated with CNBr [22], and the peptides generated were fractionated by RP–HPLC on a C_{18} column. The peak fractions were subjected to SDS/PAGE, the peptide bands transferred to polyvinylidene difluoride membrane and identified by N-terminal sequencing on Procise Protein Sequencer (Applied Biosystems, Foster City, CA, USA). The poly(vinylidene difluoride) membranes with the peptides were also incubated with different TC mAbs followed by alkaline phosphatase conjugated anti-(mouse epitope) secondary IgG. All procedures concerning electrophoresis, staining with Coomassie Brilliant Blue and western blotting were performed according to the standard protocols.

Binding of the naturally cleaved TC fragments to mAbs

Expression of TC in yeast and plants was accompanied by partial cleavage of the protein at several sites by some proteases endogenous for these systems. Protein preparations containing the peptide fragments were analyzed for immunological reactivity by western blotting and identified by N-terminal sequencing.

Binding of mAbs to synthetic peptides of TC

Two long peptides of 30 residues (P_A and P_B) were synthesized on 431A Peptide Synthesizer (Applied Biosystems): (a) KGDRLVSQLKWFLEDEKRAIGHDHKGHPHK and (b) KTQASLSGPYLTSVMGKAAGEREFWQLLRK. The underlined residues of P_A and P_B are identical to TC sequences from CNBr fragments 4 and 10–11, respectively. Two Lys residues were introduced at the ends of each peptide in order to increase the number of amino groups not relevant to the epitope structure. Purity of the isolated samples (> 95%) was verified by N-terminal sequencing. Each peptide (0.8–0.9 mg) was coupled to CNBr-activated Sepharose (1 mL) according to the standard procedure. Unreacted groups on Sepharose were blocked, and the matrix was extensively washed.

Binding of mAbs (30 $\mu g \cdot mL^{-1}$) to 0.2 mL of the peptide-resin was performed in 1 mL of 0.05 M Tris, 0.5 M NaCl, 0.1% (v/v) Tween, pH 7.5 at 20 °C. After 30 min of incubation under mild agitation the resin was washed with the same buffer (1.5 mL, 5 × 5 min) and then subjected to a secondary anti-(mouse epitiope) IgG with alkaline phosphatase (1 mL, 2 $\mu g \cdot mL^{-1}$). After 30 min of incubation, the washing procedure was repeated, and the matrix was stained for 1 min. Color development was terminated by adding 0.5 M acetate buffer, pH 4.6, whereupon the matrix was extensively washed with water. The intensity of staining was estimated visually.

Three short peptides (10–15 residues) were synthesized as described above: (a) peptide c (LALCLHQKRVHD SVV); (b) peptide d (EPFHQGHHSVD); and (c) peptide e with a disulfide bond (AL**C**LHQKR–T**C**LKRSN, connected Cys residues in bold). The peptides were used as the competing ligands during the SPR binding of TC to anti-TC Igs immobilized on the chip as described above.

Interference of the anti-TC IgG with TC functions

Binding of Cbl to TC·mAb or mAb·TC·Cbl/heparin·TC·Cbl complexes to the receptor was conducted as described earlier [16]. In another setup, interaction between TC·mAb (1 μM) and the immobilized Cbl–biotin analog was followed by SPR as described above.

Modeling of the structure

A computer based three-dimensional model of apo-TC was generated for visualization purposes on the basis of the amino acid sequence using the *ab initio* modeling procedures on the automated protein modeling server HMMSTR/ROSETTA, available at http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php. ROSETTA is a Monte Carlo Fragment Insertion protein folding program. The server also uses a hidden Markow model (HMMSTR) for local, secondary and supersecondary structure prediction, based on the I-sites library of sequence motifs that correlate with particular local structures [23,24].

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