IDENTIFICATION OF HEPARIN-BINDING SITES IN THE FIBRONECTIN TYPE III DOMAINS OF THE LEUKOCYTE COMMON ANTIGEN (CD45)

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Leukocyte common antigens (CD45) are large receptors that are abundantly expressed at the surface of all leukocytes. These receptors are type I membrane glycoproteins possessing two large C-terminal intracellular domains with protein tyrosine phosphatase activity. While the role of these enzyme domains in leukocyte signaling is well documented, the role of the N-terminal extracellular portion of CD45, composed of sequences formed by alternatively spliced exons, the cysteine rich domain, and three type III fibronectin repeats, remains unclear. The presence of fibronectin domains would predict the occurrence of heparin-binding sites, which may account for the documented affinity of CD45 for acid polysaccharides. We addressed this hypothesis using soluble recombinant proteins corresponding to the individual fibronectin domains (FN1 to FN3), and to the entire extracellular portion of CD45 (sCD45). Binding of these proteins to heparin was examined by frontal affinity chromatography. We found that while the sCD45 bound to heparin with K_d of 3.2×10^{-8} mol/l, the binding of FN2 and FN3 was somewhat weaker (K_d was 1.4 and 7.4×10^{-7} mol/l, respectively). The FN1 domain did not interact with heparin. Our results bring definitive evidence for the existence of binding sites for acid polysaccharides in the extracellular domain of CD45. These binding sites may be important for surface interactions of CD45 and for leukocyte signaling.

Keywords: Lectin-like receptors; Lectins; Antigens; Leukocytes; Carbohydrates; Oligosaccharides; Signaling; Adhesive interactions.

CD45 antigens are large surface receptors that are abundantly expressed on all nucleated cells of hematopoietic origin[1]. These receptors make about 10% of all leukocyte surface molecules, and are thus often referred to as leu-

kocyte common antigens[2]. CD45 receptors are type I membrane proteins. Short N-terminal sequence in these proteins is followed by sequences formed by three alternatively spliced exons (A, B and C) expressed only in the large isoforms of CD45 such as the maximal isoform designated as CD45RABC [3]. The cysteine-rich domain and three protein modules related to fibronectin (FN)[+] type III domains are localized in the membrane-proximal region of CD45 [4]. After the transmembrane domain there is a large intracellular portion of the molecule formed by two protein domains with enzymatic activity of the protein tyrosine phosphatase[5].

The function of the latter intracellular enzyme domains in leukocyte signaling is well documented[1,2]. On the other hand, the role of the large N-terminal extracellular part in the biology of CD45 remains a mystery, and has only recently been studied by several groups[3,6]. In our laboratory we have previously identified binding sites for complex saccharides, neutral and sialylated, in the membrane-distal extracellular part of CD45RA [7]. Since the identification of fibronectin type III domains in the extracellular part of CD45 may indicate the existence of additional saccharide-binding sites, namely those for acid polysaccharides[8,9], we decided to examine the localization of these putative binding sites experimentally. We prepared recombinant proteins encompassing the entire extracellular portion of the minimal form of CD45, CD45R0. Individual fibronectin type III domains were obtained from this protein by mild trypsin digestion. The binding of expressed and refolded proteins to a model acid polysaccharide, heparin, was then investigated by frontal affinity chromatography.

EXPERIMENTAL

Chemicals

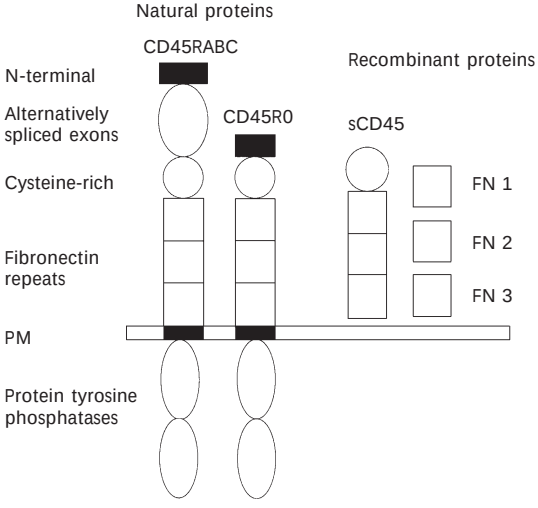
Heparin disaccharide I-S was from Sigma, and was radiolabeled with [^{3}H] as described previously[10]. Two affinity supports with varying degree of substitution with heparin were used: heparin–Sepharose (Amersham BioTech, 10 mg heparin/ml gel) and heparin immobilized to polyacrylamide (a gift from Prof. M. Tichá, Charles University, Prague, Czech Republic, 5 mg heparin/ml gel). The 30 kDa heparin-binding fragment of fibronectin, and the heparin-

+ *Abbreviations used*: BSA, bovine serum albumin; CD, cluster of differentiation (system of leukocyte surface antigens classification); FN, fibronectin; PBS, phosphate buffered saline (10 mM sodium phosphate buffer (pH 7.4) with 100 mM NaCl); PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); TBS, Tris buffered saline (10 mM Tris-HCl (pH 7.4) with 100 mM NaCl); TFA, trifluoroacetic acid.

binding fibronectin peptide, amino acids K_{1977}–T_{1991} were from Sigma. Monoclonal antibodies against CD45, clones 9.4, GAP8.3, and 4B2 were from ATCC, monoclonal antibody MEM-28 was a gift from Prof. V. Hořejší, Institute of Molecular Genetics, Prague, Czech Republic. All other chemicals were analytical grade reagents of the highest purity available from commercial sources.

Preparation of the Expression Construct

Plasmid containing the complete human CD45 insert was a gift from Prof. Mathew L. Thomas, Washington University, St. Louis, MO, U.S.A. [11]. The DNA fragment coding for the entire extracellular portion of CD45R0 starting with the cysteine-rich domain (sCD45, Cys^{226}-Lys^{575} (see Scheme 1 for the detailed description of individual proteins)) was amplified from this plasmid by PCR. We used the DeepVent DNA polymerase protocol (New England Biolabs), 5′-GATCTGTACGACGATGACGAT-AAGTGTGATGAAAAATATGCAAACATC-3′ as a forward primer, and 5′-CAC-AAGCTTTTACTACTTAGAATTATAAGATGT-3′ as a reverse primer. The amplified DNA fragment was purified by preparative agarose gel electrophoresis[12], and used for linking (by PCR) to another DNA fragment containing the histidine tag and a polylinker region of the prokaryotic expression vector pRSETB (Invitrogen); T7 promoter primer (Invitrogen) and the above reverse primer were used, respectively, as the forward and the reverse primer in the linking PCR reaction. The linked DNA fragment was again gel-purified, cleaved with restriction endonucleases *Nhe* I and *Hind* III, and the cleaved fragment ligated into the pRSETB vector that had been cleaved with the same restriction endonucleases, and digested with shrimp alkaline phosphatase (Roche). Recombinant clones were transformed into *Escherichia coli* XL-1 MRF' Blue (Stratagene), successful recombinants were selected on ampicillin (150 μg/ml), and checked by control restriction digestion of the minipreparative plasmid DNA. The expression plasmid was sequenced on both strands using the corresponding dye primers and automated DNA sequencer (ABI Prism 3100).



SCHEME 1

Preparation of Recombinant His_6-Tagged sCD45 (tsCD45)

The expression vector was transformed into *Escherichia coli* strain BL-21 (DE3) (Invitrogen). Bacterial cultures (1 l) were shaken in Erlenmayer flasks at 240 RPM and 37 °C, and grown until the optical density at 550 nm reached 0.8. Cultures were induced with 1 mM isopropyl thio-β-D-galactopyranoside and grown for another 4 h. Under these conditions, recombinant tsCD45 was precipitated in the inclusion bodies. These were isolated[13], dissolved in 8 M urea in 50 mM sodium phosphate buffer (pH 7.5) and 5 mM 2-sulfanylethan-1-ol, and the recombinant protein was purified to homogeneity on affinity column with immobilized Ni^{2+} (Ni-NTA Superflow, Qiagen) under denaturing (8 M urea) conditions. The purified protein was eluted at acidic pH (50 mM sodium phosphate buffer, pH 4.0), pH was brought back to 7.5 with 1 M NaOH, and the protein was immediately refolded by rapid dilution into the refolding buffer consisting of 20 mM Tris-HCl (pH 8.0), 1 M NaCl, 0.6 M L-arginine, 1 mM cysteamine, 0.2 mM cystamine, and 1 mM NaN_3. The refolded protein was concentrated to 10 mg/ml by ultrafiltration using cellulose membranes (Millipore) with molecular weight cut-off 100 000 (to remove large protein aggregates), and then with molecular weight cut-off 30 000. The concentrated protein was dialyzed extensively against TBS with 1 mM NaN_3 and stored as a concentrated (10 mg/ml) solution in the above buffer at 4 °C.

Preparation of sCD45 and FN Proteins

sCD45 was prepared by enterokinase (New England Biolabs) digestion of tsCD45 at room temperature for 48 h at substrate:enzyme ratio of 100:1. The completeness of enterokinase digestion was checked by SDS electrophoresis in 15% polyacrylamide gel and by N-terminal sequencing (see below). In order to cleave sCD45 further and to obtain individual FN domains, we performed mild trypsin digestion of sCD45 using substrate:enzyme ratio of 1000:1. This digestion proceeded at room temperature for 1 h and was terminated by the addition of 1 mM PMSF. The reaction mixture was acidified to pH 2.0 with concentrated TFA, centrifuged to remove any precipitated material, and separated on an analytical Vydac C-18 column (4.6 × 250 mm, 5 μm, Dionex) equilibrated in 0.1% TFA in water, and eluted with acetonitrile gradient increasing its concentration by 1% per min, up to 0.1% TFA and 70% acetonitrile in water. The elution was monitored continuously at 280 nm, protein fractions were collected manually, and aliquots were examined by SDS electrophoresis in 17.5% polyacrylamide gel followed by electroblotting onto PVDF membranes, and N-terminal sequencing (see below). Individual proteins were lyophilized, dissolved in TBS buffer as above, and stored as concentrated solutions (10 mg/ml) at 4 °C.

Analysis of CD45 and FN Proteins

The purity and identity of CD45 proteins was evaluated using several methods. SDS electrophoresis under both reducing and nonreducing conditions was performed in the buffer system of Laemmli[14]. The separated proteins were also blotted onto PVDF membrane (Immobilone P, Millipore) and 8 cycles of automated Edman degradations (Protein Sequencer LF3600D, Beckman) were performed to verify the N-terminal sequence[15]. Native size and monomeric status of the proteins were routinely evaluated using gel filtration on Superdex 75 HR and Superdex 200 HR columns (Amersham BioTech). The correct size of the proteins was evaluated by MALDI mass spectrometry[15]. Correct refolding of the individual proteins was also checked immunochemically by following the reactivity of the denatured

and the refolded proteins with conformation-sensitive and nonsensitive monoclonal antibodies against CD45 [16,17] as described previously[10]. Briefly, 1 μg of CD45 proteins in 50 μl of PBS was added in triplicate wells (Immulon 4, Dynatech) and incubated at 4 °C for 16 h. Wells were washed with PBS, blocked with 1% BSA in PBS, and incubated with primary murine antibodies (10 μg protein/ml) at the indicated temperature for 6 h. Wells were washed with PBS and binding of the primary antibody was detected after incubation with goat antibodies against mouse immunoglobulin labeled with $Na^{125}I$ (specific activity 10^6 cpm/mg protein) for 30 min. After the final washing with PBS, radioactivity in individual wells was measured by liquid scintillation counting (Microbeta, Wallac). Protein in control wells were denatured with 6 M guanidine–HCl and 10 mM DTT for 1 h prior to washing and incubation with the primary antibodies.

Affinity Techniques

For sensitive detection of sCD45 proteins during frontal affinity chromatography, these proteins were radioiodinated using the Iodogen protocol (Pierce) and carrier-free $Na^{125}I$ (Amersham BioTech) to a final specific radioactivity of 10^5 Bq/μg protein. Frontal affinity chromatography was performed and the results were evaluated as previously described[18,19]. Briefly, in this method a solution of one of the interacting substances, A, is continuously applied at a constant concentration, $[A]_0$, to a column on which its counterpart, B, is immobilized[18]. The volume of the elution front, V, is measured as a volume of eluent corresponding to $[A]_0/2$. When there is a specific interaction with the ligand immobilized on the column, the elution volume V is larger than the elution volume of control compound having no interaction, V_0. From the simple measurements of elution volumes, both dissociation constant, K_d, and the amount of total interacting ligand, B_t, can be calculated using an equation $1/[A]_0(V - V_0) = (K_d/B_t)(1/[A]_0) + (1/B_t)$ [19]. Affinity supports were packed into 1 ml columns prepared from glass pipettes, and the columns were equilibrated in TBS. Individual proteins were dissolved in this buffer at the initial concentrations indicated at each experiment. Ovalbumin and lysozyme were used as control (noninteracting) proteins. In some experiments, direct binding assays employing equilibrium dialysis were performed essentially as described previously[10].

RESULTS

Preparation and Folding of Soluble CD45

Employing the naturally occurring CD45 proteins in heparin-binding experiments would be very complicated because of their large size, hydrophobic nature (membrane proteins), and abundant glycosylation (both N- and O-linked) in the extracellular part of their molecules. In particular, we were concerned that the latter property of CD45 might obscure the presence of binding sites for saccharides due to the blocking by the endogenous glycosylation. Therefore, we preferred to produce the extracellular part of CD45 as well as the individual FN type III domains initially as nonglycosylated proteins using a prokaryotic expression in *Escherichia coli*.

When preparing a protein corresponding to the entire extracellular part of CD45, we have selected a minimal splice variant, CD45R0 (cf. Scheme 1 for CD45 forms). We transferred the corresponding DNA fragment coding for Cys^{226}-Lys^{575} into the prokaryotic expression vector, and verified the resulting expression plasmid by DNA sequencing. Because the protein we wanted to obtain is rather large for prokaryotic expression, we had to test several bacterial strains for optimal production. Only the strain BL21 (DE3) turned out to be capable of producing the protein in amounts suitable for refolding trials. Because the induced protein precipitated into inclusion bodies, numerous protocols had to be tested to obtain well refolded, biologically active proteins. Our refolding attempts were successful only when the protein was purified before refolding, while the attempts to refold partially purified protein resulted in its complete precipitation (results not shown). Rapid dilution into a solution containing both high concentrations of salt and L-arginine, and supplemented with the cysteamine/cystamine redox buffer provided the best yields of the refolded protein. The course of the refolding was followed by immunochemical tests based on the previously described conformation sensitive monoclonal antibodies (Table I).

TABLE I
Thermal stability of soluble CD45 proteins and the derived FN domains examined using conformation-sensitive monoclonal antibodies[a]

	T, °C	MEM-28		9.4		GAP8.3		4B2	
sCD45	4	7580[b]	7720[c]	7553[b]	110[c]	7542[b]	132[c]	7620[b]	133[c]
	37	7610[b]	7493[c]	7620[b]	103[c]	7661[b]	117[c]	7710[b]	115[c]
FN1	4	315[b]	416[c]	135[b]	217[c]	4350[b]	212[c]	215[b]	217[c]
	37	317[b]	378[c]	120[b]	210[c]	4371[b]	225[c]	220[b]	228[c]
FN2	4	3215[b]	3229[c]	3223[b]	257[c]	217[b]	240[c]	210[b]	205[c]
	37	3317[b]	3310[c]	3215[b]	264[c]	238[b]	242[c]	217[b]	200[c]
FN3	4	357[b]	416[c]	203[b]	210[c]	213[b]	210[c]	8407[b]	207[c]
	37	320[b]	310[c]	238[b]	216[c]	220[b]	217[c]	8520[b]	223[c]

[a] Immunoreactivity expressed as cpm of the bound secondary antibodies averaged from triplicate experiments. [b] Native conditions. [c] Denaturing conditions.

Preparation and Characterization of FN Domains of CD45

In order to prepare proteins suitable for saccharide-binding studies, it was first necessary to remove the N-terminal His_6 tag known to cause nonspecific binding to saccharides[20]. This cleavage was achieved very efficiently using enterokinase as shown by Edman degradation data and mass spectrometry (Table II). For the preparation of smaller proteins corresponding to the individual FN type III domains, two strategies were possible. The first strategy would require to construct a series of separate expression vectors for each of these domains. However, it was known from the literature that the expression of these small proteins turned out to be very difficult in both the prokaryotic and the eukaryotic expression systems[21]. Therefore, we assumed that the large sCD45 proteins corresponding to the entire extracellular portion of CD45 had three well refolded FN domains that might be resistant to mild proteolysis. We have found that optimal conditions for this experiment included a high substrate:enzyme ratio (1000:1), short digestion times (less than 1 h), and lowered temperature (i.e. room

TABLE II
Summary of physicochemical properties of the recombinant CD45 proteins

Protein	Description	Fraction (Vydac)	N-terminal sequence	M.w. (theor)	M.w. (MALDI MS)
tsCD45	Extracellular, plus His_6tag	[a]	MRGSHHHH	43904	44120
sCD45	Extracellular, Cys^{226}-Lys^{575}	[a]	XDEKYANI	40358	40520
FN1	N-terminal FN Phe^{304}-Lys^{384}	2	FQLHDXTQ	9681	9692
FN2	middle FN Thr^{395}-Lys^{479}	1	TDFGXPGE	10923	10933
FN3	C-terminal Ser^{480}-Lys^{575}	3	SAPPSQVW	11174	11181
FN2b	truncated FN2	4	SEAXHQGV	9274	9276
FN3b	truncated FN3	5	XRPXRDXN	8657	8659

[a] Not applicable.

temperature rather than 37 °C). Under these conditions, the N-terminal cysteine-rich domain of CD45 appeared to be completely digested, but the FN domains were only disconnected, and could be then separated by chromatography on reverse-phase column. Using even milder conditions for trypsin digestion, we experienced very low yield of the individual domains. On the other hand, more extensive trypsin cleavage led eventually to a complete degradation of the entire protein, including the FN domains. Under the optimal conditions described here, FN domain 2 and 3 already started to be partially degraded at the secondary trypsin cleavage sites, as shown by SDS electrophoresis, Edman degradation and mass spectrometry (Fig. 1 and Table II, proteins FN2b and FN3b). N-Terminal sequencing and mass spectrometry was used to verify the extent of trypsin cleavage in FN proteins, and this was in a good agreement with the suggestions coming from multiple sequence alignments[4]. On the other hand, the analysis of individual FN proteins by SDS electrophoresis (Fig. 1), which was used to check the purity and the yields of these proteins, provided somewhat unusual values of molecular weights for at least two of the proteins prepared here (FN1 and FN3, cf. Table II with Fig. 1, lanes 2 and 4). The reason for this discrepancy is currently not known since the molecular weights estimated by MALDI mass spectrometry provided in all instances values corresponding very well to the anticipated amino acid sequences. This excludes the possibility that the above discrepancy might be accounted for by unusual protein modifications (such as phosphorylations), known to distort the mobility of proteins during SDS electrophoresis.

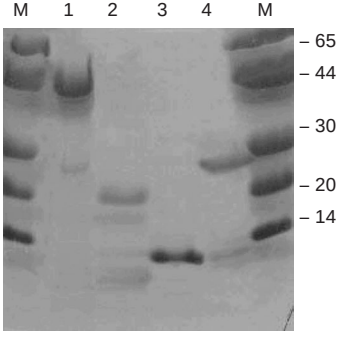


FIG. 1
SDS electrophoresis of the CD45-related proteins used in this study. M, protein marker consisting of (from top to bottom) bovine serum albumin (65 kDa), ovalbumin (44 kDa), carbonic anhydrase (30 kDa), soya trypsin inhibitor (20 kDa), and lysozyme (14 kDa); 1 sCD45, 2 FN1, 3 FN2, 4 FN3

Binding of CD45 to Heparin Examined by Frontal Affinity Chromatography

Frontal affinity chromatography is a well documented and simple method suitable for initial characterization of weak biological interactions. In this method, several concentrations of the ligand are first immobilized onto a solid support, and affinity columns with varying ligand densities are prepared. Onto such columns, a solution of binding protein is applied in several different concentrations. The protein that has no interaction with the immobilized ligand is always eluted in the void (V_0) volume independently on its concentration, and on the concentration of the immobilized ligand. If the binding protein has affinity for the immobilized ligand, we observe a concentration-dependent retardation of the elution front. From mathematical evaluation of the elution curves, both the value of dissociation constant and the amount of biologically active immobilized ligand can be calculated. We used ovalbumin and lysozyme as control protein since they have no interaction with heparin, and the range of their molecular weights covers well that of CD45 proteins used here. The elution volumes for both control proteins was nearly equal, and very close to the total column volume indicating only negligible gel filtration effects (V_0 was calculated as the averaged value for the two proteins; data not shown). Of the CD45 proteins tested, sCD45, FN2 and FN3 all displayed a concentration-dependent retardation of the elution front on heparin–Sepharose columns (Fig. 2, panels a, c and d). On the other hand, very little retardation was observed for the FN1 protein (Fig. 2, panel b). The results obtained with the heparin immobilized to polyacrylamide were similar except for the higher elution volumes due to a lower degree of substitution (data not shown). The mathematical evaluation of the binding parameters is based on the linear function obtained when $1/[A]_0$ $(V - V_0)$ is plotted against $1/[A]_0$, where $[A]_0$ is the concentration of the applied binding protein, V is the elution volume of the interacting protein, and V_0 is the elution volume of protein having no specific interaction (Fig. 3a). All experimental data obtained in this study fitted well into the above described linear functions (graphs for sCD45, FN2 and FN3 are shown in Fig. 3b, 3c and 3d, respectively). The numerical values extracted from these plots are summarized in Table III. These data indicate high-affinity interactions of sCD45, FN2 and FN3 proteins with the heparin column, and provide an estimation of the amount of biologically active ligands present in these columns. As a positive controls in these studies, the interactions of both the 30 kDa fragment from fibronectin and of the fibronectin peptide K_{1977}–T_{1991} that are known to bind to heparin[22,23] with high affinity were tested. Their interaction with both hep-

arin matrices under the experimental conditions (TBS) employed here were extremely strong, leading to elution volumes that were difficult to evaluate. Therefore, we performed direct binding assays[10] with these compounds using much smaller heparin fragment in the form of a trisulfated disaccharide I-S (ΔUA-2S-α1–4GlcNS-6S). The results of these studies indicated much stronger interaction of authentic fibronectin fragments with heparin compared to FN modules derived from CD45 (Table III): even with the small disaccharide values of K_d in nanomolar range have been obtained.

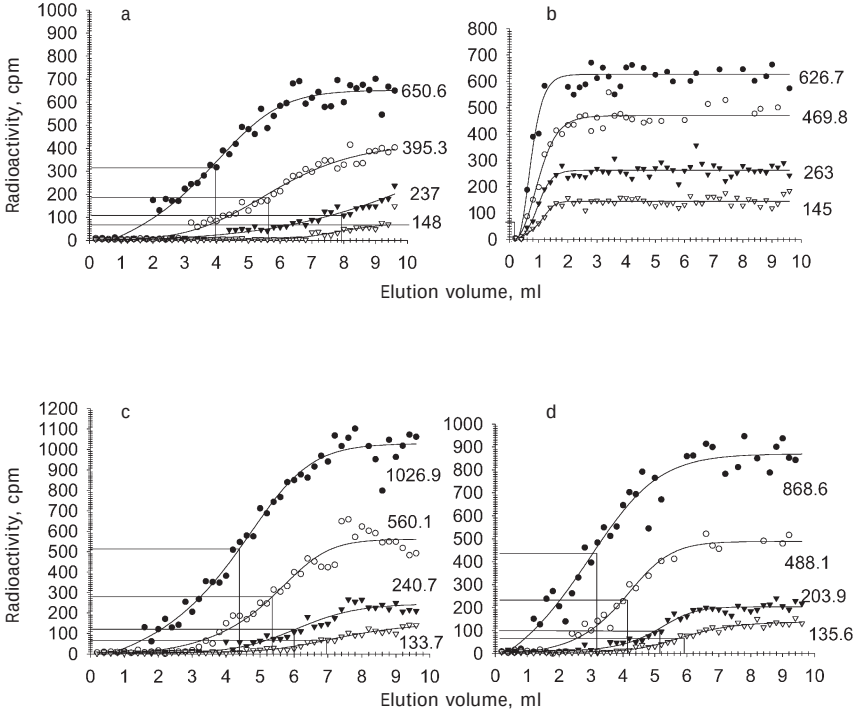


FIG. 2

Experimental data from the frontal affinity chromatography of a sCD45 (● 137 pmol/l (V = 3.88 ml), ○ 68 pmol/l (V = 5.57 ml), ▼ 34 pmol/l (V = 7.85 ml), ▽ 17 pmol/l (V = 10.5 ml*)); b FN1 (● 160 pmol/l (V = 0.75 ml), ○ 80 pmol/l (V = 1.00 ml), ▼ 40 pmol/l (V = 0.98 ml), ▽ 20 pmol/l (V = 1.08 ml)); c FN2 (● 150 pmol/l (V = 4.40 ml), ○ 75 pmol/l (V = 5.36 ml), ▼ 38 pmol/l (V = 6.04 ml), ▽ 19 pmol/l (V = 6.94 ml)); d FN3 (● 145 pmol/l (V = 3.04 ml), ○ 72 pmol/l (V = 4.06 ml), ▼ 36 pmol/l (V = 5.05 ml), ▽ 18 pmol/l (V = 5.79 ml)). The exact elution volume for the lowest concentration of sCD45 was difficult to read off (marked with an asterisk)

TABLE III
Summary of the binding data for the interaction of CD45 with heparin

Protein	Description	K_d, l nmol^{-1}	B_t, nmol
sCD45	entire extracellular part	32	0.45
CD45, FN1	membrane distal FN	[a]	[a]
CD45, FN2	middle FN	140	0.90
CD45, FN3	membrane proximal FN	740	0.43
Fibronectin domain	heparin-binding, Sigma	1.5	[b]
Fibronectin peptide	heparin-binding, Sigma	4.2	[b]

[a] Not detectable. [b] Not determined.

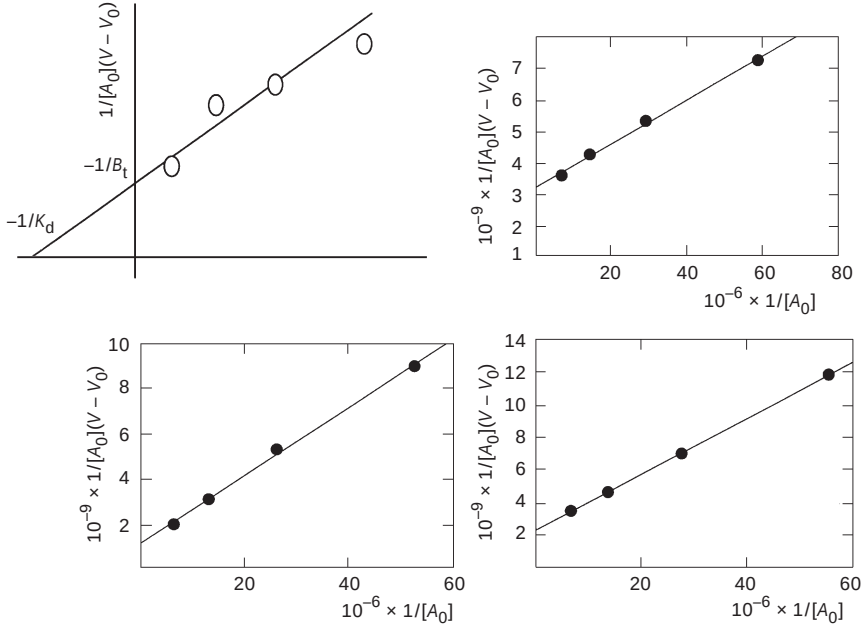


FIG. 3
Plots for the evaluation of the binding parameters from the elution profiles of frontal affinity chromatography shown in Fig. 2: a general scheme, b sCD45, c FN2, d FN3. There has been no specific binding for FN1

DISCUSSION

CD45 is one of the most abundant, and also most complex leukocyte surface receptors. While the participation of this molecule in leukocyte signaling has been at least partially clarified after the discovery of the protein tyrosine phosphatase activity associated with the intracellular domains of this molecule, the role of the large extracellular part of CD45 in numerous interactions of this receptor remains poorly understood. We have previously shown the occurrence of a specific binding site for complex saccharides in the extracellular part of CD45, namely in the region formed by the alternatively spliced exons in the long CD45 isoform, CD45RA [7]. The recent identification of FN type III modules in the extracellular part of CD45 pointed to a possibility that acid saccharides, well documented fibronectin ligands[9,22,23], might interact with CD45. This possibility was further suggested by the earlier studies detecting the interaction of the entire CD45 from detergent lysates of lymphocytes with acid polysaccharides[8]. We have thus decided to test these possibilities experimentally using various segments of the extracellular part of CD45 expressed in the prokaryotic expression system. The use of this particular expression system was critical since the natural CD45 isoforms or CD45 expressed in the eukaryotic expression systems are heavily glycosylated, which might obscure the identification of binding to saccharides due to their blocking by the endogenous glycosylation. Since there have been reports in the literature about the difficulties in the expression of individual FN domains of CD45 in both prokaryotic and eukaryotic expression systems, we have chosen to prepare a soluble CD45 protein corresponding to the entire extracellular portion of the minimal form of CD45 (CD45R0), and to obtain individual domains from this large protein using mild proteolysis. This approach has been successful in that we managed to find conditions for the controlled digestion of CD45 with trypsin, which allowed to prepare all three FN type III domains in reasonable yields.

The results of binding studies performed using a well documented and reproducible methodology based on frontal affinity chromatography clearly show that nonglycosylated extracellular part of CD45, sCD45, binds to heparin with high affinity as indicated from the micromolar values of the dissociation constant. This binding is most probably based on the cooperation of two heparin-binding sites detected in the individual FN type III domains, domain 2 and domain 3. It is also worth mentioning that the biologically active amounts of heparin-related ligands make a minor portion of the total amount of the immobilized heparin: while there has been about 10^{-6} mol

of this polysaccharide immobilized in the affinity column, only nanomolar amounts were effectively interacting with CD45. This clearly indicates that the biologically active compound responsible for the interaction of heparin with CD45 can represent only a small fraction (about 0.1%) of this polysaccharide. The identification of the exact structure of this compound using the previously described methodology[24], which may be important for understanding the biological interactions involving extracellular CD45 sequences[25–27], will be an interesting subject for future studies.

It would appear from the data presented here that the interaction of fibronectin modules of CD45 with heparin is much weaker than found for the modules derived from the prototype member of this family, fibronectin. This may be related to the fact that CD45 contains a special class of FN domains that are rich in cysteines but do not contain an extensive array of the heparin-binding peptides that are characteristic for fibronectin[4,22]. On the other hand, our preliminary results with glycosylated CD45 produced in eukaryotic expression systems indicate that glycosylation does not completely abbrogate the binding to heparin[28]. Therefore, we assume that our findings obtained with bacterial proteins are also valid for the native, glycosylated CD45 produced in the eukaryotic expression systems, and for CD45 at the surface of leukocytes.

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