Characterization of the Calcium Site in Two Complement-like Domains from the Low-Density Lipoprotein Receptor-Related Protein (LRP) and Comparison with a Repeat from the Low-Density Lipoprotein Receptor†

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ABSTRACT: Calcium is required for the binding and endocytosis of protein ligands by the low-density lipoprotein receptor-related protein (LRP) and other members of the low-density lipoprotein (LDL) receptor family. Calcium binding sites are thought to be present in the complement-like repeats that occur in clusters in all members of this receptor family. We have expressed two such complement-like repeats, CR3 and CR8, from an α_2-macroglobulin−proteinase ligand binding region of LRP, as well as repeat 1 from the LDL receptor and examined the metal binding properties and resulting structural changes of these three repeats using changes in tryptophan and terbium fluorescence and perturbation of [^{1}H-^{15}N]-HSQC NMR spectra of the ^{15}N-labeled domains from LRP. We found that all three domains contain a tight calcium binding site at physiological pH and that calcium binding results in a major structural rigidification. Changes in tryptophan fluorescence and tryptophan-sensitized terbium fluorescence indicate that the calcium binding sites are located in homologous regions in all of the repeats. Differences in the details of the perturbations, as well as in the pH dependence of calcium binding, show, however, that each metal site is distinct.

The low-density lipoprotein receptor-related protein (LRP)[1] is a member of a family of receptors that are both structurally and functionally related to the low-density lipoprotein receptor (LDLR) (*1*). Each of these proteins contains multiple copies of epidermal growth factor-like and complement-like repeats, so named for their similarity to epidermal growth factor and a repeat found in complement components C8 and C9, respectively, as well as repeats that contain a YWTD motif. LRP is the largest member of this receptor family, being synthesized as a 600 kDa precursor that is cleaved into a heterodimer that consists of a C-terminal 601 residue light chain (β) and an N-terminal 3923 residue heavy chain (α) (*2*). The β chain comprises a small cytoplasmic domain, a single transmembrane helix, and an extracellular domain that has seven copies of the EGF-like repeat. The α chain contains an additional 15 single or tandem EGF-like repeats interspersed among 31 complement-like repeats grouped in 4 clusters of 2−11 repeats and 7 clusters of theYWTD-containing repeats.

Like LDLR and VLDLR, LRP can bind to certain apolipoproteins and to lipoprotein particles that contain them. In addition, however, LRP binds to a very wide array of apparently unrelated ligands including certain serpin−proteinase complexes (3−5), α_2-macroglobulin−proteinase complexes (*6*), β-amyloid peptide (*7*), lactoferrin (*8*), *Pseudomonas* endotoxinA (*9*), and the receptor-associated protein (RAP) (*10*). LRP is the principal clearance receptor for α_2-macroglobulin−proteinase complexes and some, if not all, serpin−proteinase complexes. It is possible that the ability of LRP to bind such a diverse array of ligands is related to the presence of many slightly different complement-like repeats, which may form multiple, slightly different ligand binding sites. Three studies have implicated the second cluster of complement-like repeats (counting from the N-terminus) as a region of interaction with α_2M−proteinase complexes (*11*−*13*). The 8 repeats present in this cluster are about 42 residues long and contain 6 conserved cysteines that form 3 disulfide bridges. Despite the similarity in length and cysteine content of these repeats, there are no other residues, other than four carboxyl ligands that are ligands to the calcium ion, that are absolutely conserved in the cluster of eight repeats that contain the α_2M−proteinase binding site (Figure 1). There are, however, five positions where a residue is conserved in seven of the eight repeats. Although no structure of any complement-like repeat has been reported

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[1] Abbreviations: α_2M, α_2-macroglobulin; RBD, receptor binding domain; LRP, low-density lipoprotein receptor-related protein; LDLR, low-density lipoprotein receptor; VLDLR, very low density lipoprotein receptor; CR3 and CR8, third and eighth complement-like repeats, respectively, from LRP; LB1, LB2, and LB5, first, second, and fifth complement-like repeats, respectively, from LDLR; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; TFA, trifluoroacetic acid; HSQC, heteronuclear single quantum coherence; EGF, epidermal growth factor.

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Repeat																														
CR3			*G*	*S*	P	P	Q	C	Q	P	G	E	F	A	C	-	-	A	N	S	R	C	I	Q	E	R	**W**	K	C	
CR8			*G*	*S*	P	G	G	C	H	T	D	E	F	Q	C	-	R	L	D	G	L	C	I	P	L	R	**W**	R	C	
LB1	*G*	*S*	A	V	G	D	R	C	E	R	N	E	F	Q	C	-	-	Q	D	G	K	C	I	S	Y	K	**W**	V	C	
LB2		*G*	*S*	L	S	V	T	C	K	S	G	D	F	S	C	G	G	R	V	N	R	C	I	P	Q	F	**W**	R	C	
LB5							P	C	S	A	F	E	F	H	C	-	-	L	S	G	E	C	I	H	S	S	**W**	R	C	

Repeat																				
CR3	**D**	**G**	**D**	N	**D**	C	L	D	N	S	**D**	**E**	A	P	A	L	C	H	Q	H
CR8	**D**	**G**	**D**	T	**D**	C	M	D	S	S	**D**	**E**	K	S	-	-	C	E	G	V
LB1	**D**	**G**	S	A	**E**	C	Q	D	G	S	**D**	**E**	S	Q	E	T	C	L	S	V	T
LB2	**D**	**G**	Q	V	**D**	C	D	N	G	S	**D**	**E**	Q	G	-	-	C			
LB5	**D**	**G**	G	P	**D**	C	L	D	N	S	**D**	**E**	E	N			C	A		

FIGURE 1: Comparison of the primary structures of selected complement-like repeats from LDLR and LRP. Sequences for CR3 and CR8 are compared with those of the three domains from the LDL receptor for which structural information is available: LB1, LB2, and LB5. The sequences are aligned at the positions of the six conserved cysteines. The four carboxyl ligands that coordinate the metal ion in LB5, and that are conserved in all eight repeats of the second cluster of LRP and in six of the seven repeats of LDLR, are shown in boldface type, as are the glycine and tryptophan that coordinate the calcium in LB5 through their backbone carbonyls. The two additional aspartates that are present in CR3 and CR8, in the sequence DxDxD, and that are expected to be in a position to also coordinate the metal ion, are shown in boldface and are underlined. Four other residues that are highly conserved, both within these five repeats and more generally in LDLR, and in the second cluster of complement repeats in LRP are shaded.

for LRP, two NMR structures (LB1 and LB2) (*14, 15*) and one crystal structure (LB5) (*16*) have been determined for repeats from the related LDL receptor. It has been proposed, based on the crystal structure of one such repeat from LDLR with calcium bound, that familial hypercholesterolemia may result from mutations of calcium ligands that adversely affect binding of calcium to the complement-like repeat and so reduce binding of the receptor to the α-helical, lysine-containing binding site of apolipoprotein E (*16*). It has been shown that calcium is also required for binding of α_2M−proteinase complexes to LRP and that this involves calcium binding to LRP rather than to α_2M (*17*). This calcium-mediated binding may well involve an equivalent type of binding site in each of the complement-like repeats.

Given the large differences in primary structure between the various complement-like repeats (Figure 1), the much broader protein ligand specificity of LRP compared with LDLR or VLDLR, and the absence of any structural information on repeats from LRP, it will be necessary to have structures of specific LRP-derived complement-like repeats to understand specific LRP−ligand interactions. We have therefore initiated a program of study of the repeats from LRP to determine the similarities and differences between individual repeats within LRP and between repeats from different receptors of the family. In this paper we report the effects of calcium binding on the structures of repeats CR3 and CR8 from LRP and the characterization of the calcium binding sites, and make comparisons with the first complement-like repeat from LDLR, LB1, whose structure has already been determined by NMR (*14*). The repeats from LRP are the first and sixth from the cluster of eight that contains the α_2M−proteinase binding site and the third and eighth such repeats, respectively, from the N-terminus. They are therefore designated CR3 and CR8.

MATERIALS AND METHODS

Cloning, Expression, and Folding of CR3, CR8, and LB1. For each construct, four overlapping oligonucleotides (approximately 50-mers) encoding a 5′ *Bam*HI site, the ligand binding domain, and a 3′ stop codon, followed by an *Xba*I site were purchased (Gibco). After purification by thin-layer chromatography (SurePure, USB), the oligonucleotides were annealed pairwise (1+2 and 3+4) and made double-stranded using Sequenase. The two pieces of double-stranded DNA were joined and amplified by high-fidelity PCR (Elongase, Gibco BRL) using the outer oligonucleotides (1 and 4) as primers. The fragment was cloned into pGEX-2T(XbaI) and the identity of the construct confirmed by sequencing.

Unlabeled complement repeats were expressed in *E. coli* BL21(DE3). Expression was induced with 1 mM IPTG at $OD_{600} = 0.60$, and the cells were grown for 4 h at 37 °C in rich medium. ^{15}N-labeled CR3 and CR8 were expressed in minimal medium containing 0.6% Basal Medium Eagle Vitamin solution (Gibco), 1 g L^{-1} $(^{15}NH_4)_2SO_4$, and 2 g L^{-1} unlabeled glucose. Optimal yield was achieved by harvesting the cells 6 h after induction. GST−complement repeat fusion proteins were purified according to (*18*), cleaved with thrombin (1/1000 w/w, 5−10 min at 20 °C), and rechromatographed on GSH−Sepharose. The nonbinding fraction was further purified by gel filtration on Sephadex G-50F in 5% formic acid, lyophilized, and dissolved in 6 M guanidinium chloride, 50 mM Tris-HCl, 1mM dithiothreitol, pH 8.5. The sample was diluted to approximately 200 μg mL^{-1} with folding buffer before folding in vitro by dialysis against 50 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$, 1 mM GSH, and 0.5 mM GSSG, for 24 h at room temperature under oxygen-free conditions (*19*). Folded ligand binding domains were purified by reverse-phase HPLC on a Nucleosil C-18 column (4.6 × 250 mm) operated at a flow rate of 0.75 mL/min using a linear gradient of 0.1% trifluoroacetic acid (TFA) (A) and 90% acetonitrile, 0.075% TFA (B) (20−50% B) over 23 min at room temperature. The eluting domains were followed by the absorbance at 280 nm.

NMR Spectroscopy. NMR spectra were recorded at the University of Illinois at Chicago on a Bruker DRX600 equipped with a pulsed-field-gradient accessory and operating at 600.13 MHz for ^{1}H. NMR data were processed and analyzed using Triad 6.3 software (Tripos, Inc., St. Louis, MO). Lyophilized CR3 and CR8 were dissolved in 20 mM CD_3COOD, 10% D_2O, pH 5.5, and the pH was maintained at 5.5 by addition of NaOH. The final concentration of protein was 100 μM. 2D [^{1}H-^{15}N]-HSQC spectra (*20*) were recorded at 298 K in the absence or presence of 10 mM $CaCl_2$. The center frequencies were 4.70 and 118 ppm for

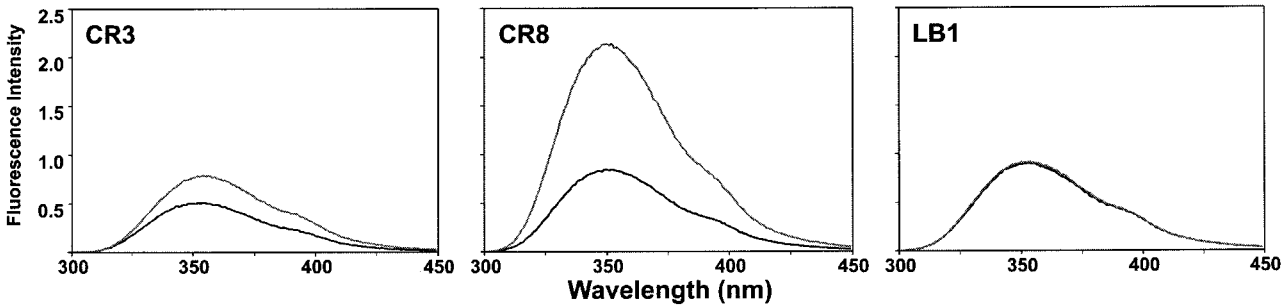

FIGURE 2: Tryptophan fluorescence emission spectra of CR3, CR8, and LB1 in the absence (solid line) and presence (shaded line) of 10 mM $CaCl_2$. From left to right, CR3, CR8, and LB1. Spectra were recorded at 25 °C in 20 mM HEPES buffer, pH 7.4, containing 100 mM NaCl.

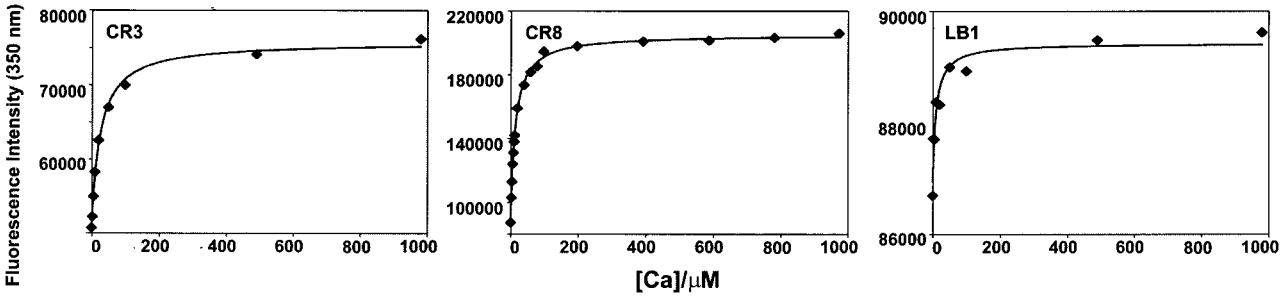

FIGURE 3: Saturable binding of Ca^{2+} at pH 7.4 to complement-like repeats, monitored by the change in tryptophan fluorescence at 350 nm. The solid line is the fit to a single binding site.

^1H and ^{15}N, respectively. Sensitivity enhancement gradient pulses were employed (*21*, *22*).

Fluorescence Spectroscopy. Fluorescence spectra were recorded on an SLM8000 spectrofluorimeter. Protein concentration was 1 μM. Spectra were recorded at 25 °C using slits of 4 nm for both excitation and emission. For metal ion titrations monitored at constant wavelength, an emission slit of 8 nm was used, with excitation at 280 nm and detection at 350 nm for tryptophan fluorescence and excitation at 280 nm and detection at 545 nm for terbium fluorescence. Dissociation constants were obtained by fitting data to a single binding site model by nonlinear least-squares fitting, using the program Scientist (MicroMath, Salt Lake City, UT).

Materials. Oligonucleotides and enzymes were from Gibco BRL. SurePure was from USB, and Sequenase was from Amersham. Plasmid pGEX-2T(XbaI) was a kind gift of Dr. Robert Costa, UIC. Sepharose 4B, Sephadex G-50 F, and Mono Q (HR5/5) were from Pharmacia. Nucleosil 100-5 C-18 was from Machery-Nagel. Gadolinium oxide and terbium peroxide were from Sigma. These were dissolved in concentrated HCl to give the trichloride. 99.997% $CaCl_2$ was from Johnson Matthey, Ltd. $(^{15}NH_4)_2SO_4$ was from Cambridge Isotope Laboratories Inc.

RESULTS

Tryptophan Fluorescence Changes Upon Calcium Binding. Both CR3 and CR8 contain only a single, homologous tryptophan. This tryptophan is also conserved between LB1, LB2, and LB5, but in the context of different flanking residues in each case (Figure 1). In the crystal structure of Ca-LB5, the carbonyl oxygens of the tryptophan, as well as of glycine four residues C-terminal, are ligands to the calcium. The tryptophan fluorescence emission spectra of CR3, CR8, and LB1 all had maxima at 350 nm consistent with a solvent-accessible environment for the tryptophan (Figure 2), with relative intensities of 1:1.70:1.75, respectively. Addition of calcium caused enhancements in intensity of 59% for CR3 and 152% for CR8, but only about 3% for LB1. In each case the increase in intensity was saturable, consistent with a single specific calcium binding site in each domain (Figure 3). Estimates of the dissociation constants obtained from fitting the titration data gave values of 24, 13, and 7 μM for CR3, CR8, and LB1, respectively. There was a small red shift ($\sim$2 nm) in the emission maximum for CR3 and CR8, but no change for LB1. These results suggest that CR3 and CR8 possess a tight calcium binding site that is similar in location to that present in LB1 and LB5, though with distinct effects of calcium binding for each repeat.

Sensitized Terbium Emission Spectra. Terbium emission spectra were obtained for CR3, CR8, and LB1 containing saturating levels of terbium (Figure 4). This lanthanide is a good replacement for calcium, despite the higher charge of the lanthanide, and is one that can give sensitized fluorescence emission as a result of energy transfer from a nearby excited tryptophan or tyrosine side chain. Detection of sensitized terbium emission upon specific excitation of tryptophan at 280 nm is thus evidence for proximity of the tryptophan (there are no tyrosines in these repeats). Consistent with tryptophan being very close to the terbium binding site in each protein, there was a large increase in terbium fluorescence upon binding terbium to both CR3 and CR8 (Figure 4), with the expected concomitant decrease in tryptophan emission intensity. Binding of terbium to LB1 gave a large increase in terbium emission, accompanied by an *increase* in tryptophan fluorescence, suggesting that terbium caused two opposing effects on tryptophan intensity: an enhancement due to binding and a smaller reduction due to energy transfer to terbium.

As with the perturbation of tryptophan fluorescence by calcium, increase in terbium fluorescence was a saturable

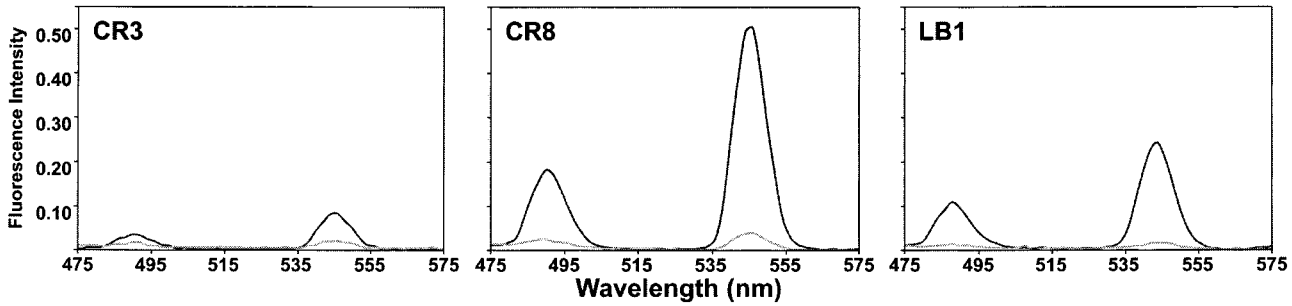

FIGURE 4: Terbium fluorescence spectra resulting from energy transfer from tryptophan directly excited at 280 nm. Spectra are for 20 μM $TbCl_3$ in the presence of 1 μM complement-like repeat before (solid line) and after (shaded line) addition of 2 mM Ca^{2+} to displace terbium. In the absence of protein, 20 μM $TbCl_3$ gave no detectable emission when excited at 280 nm. Terbium gives more than one emission band, though the more intense band at 545 nm was used for monitoring metal ion binding. Panels are, left to right, CR3, CR8, and LB1.

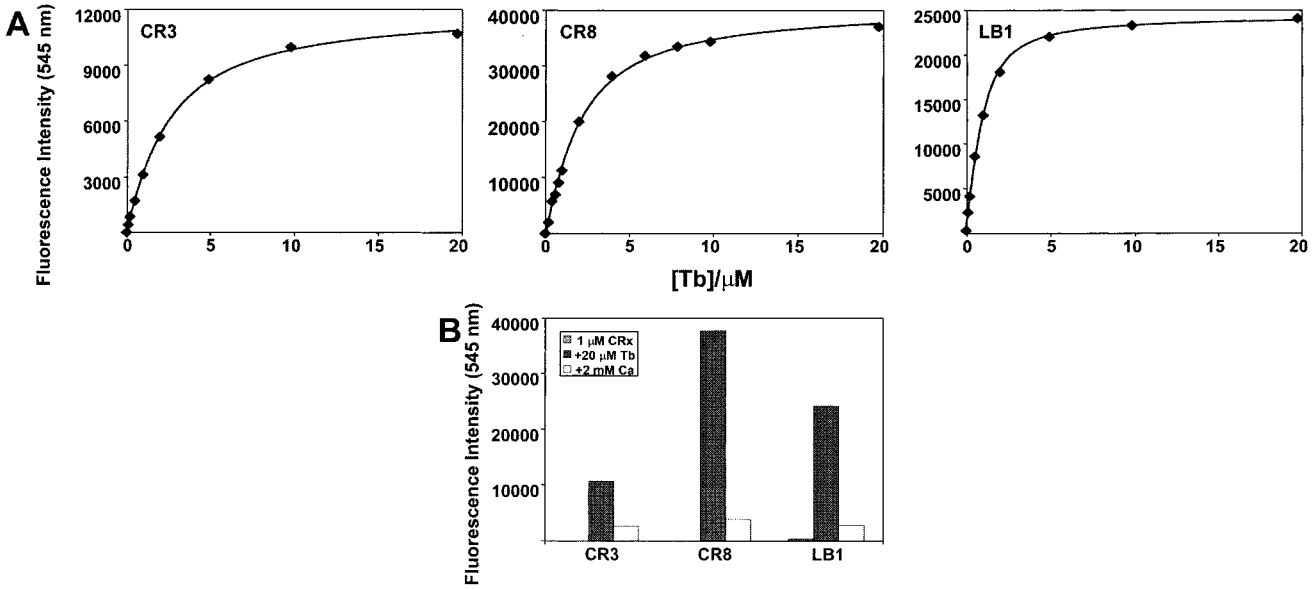

FIGURE 5: Saturable enhancement of terbium fluorescence and displacement of Tb^{3+} by Ca^{2+}. (A) Increase in sensitized terbium fluorescence at 545 nm. The solid line is the best fit to the data. (B) Effect of 2 mM added calcium on terbium fluorescence for 1 μM complement-like repeats in the presence of 20 μM $TbCl_3$ (the final point of the titration shown in panel A).

effect for all three repeats, consistent with a single tight terbium binding site (Figure 5A), and could be reversed by addition of excess calcium, showing that terbium was binding to the same site as calcium (Figure 5B). The enhancement was about 4-fold greater for CR8 than CR3, which may be due to more efficient transfer in CR8. Dissociation constants for the binding of terbium, obtained from nonlinear least-squares fitting of the titration data, were 2.0, 1.7, and 0.4 μM for CR3, CR8, and LB1, respectively. This is the same order of affinity as for calcium binding. The higher affinity of terbium almost certainly results from the higher positive charge (3+) compared with calcium (2+). These results suggest that the single calcium site in each domain is very close to the conserved tryptophan, as it is in LB5, but that the position relative to the indole ring may differ between LB1 and CR3/CR8.

Gadolinium Binding to CR3, CR8, and LB1. It has previously been claimed, based on the absence of specific gadolinium-induced broadening effects on the ^1H NMR spectrum of LB1, that gadolinium does not bind to the calcium site of LB1 or induce the native calcium-dependent fold (*23*). Since we had found above that terbium, which is adjacent to gadolinium in the periodic table, binds tightly to LB1 at the calcium site, we reinvestigated whether gadolinium might bind specifically to LB1, using perturbation of tryptophan fluorescence as a probe of gadolinium binding to the calcium site. Consistent with the specific binding of terbium, we found that gadolinium bound in a saturable manner and gave a large increase in tryptophan fluorescence (Figure 6), that could be reversed by addition of calcium. The dissociation constant obtained from a fit of the titration data was 0.3 μM, which is very similar to the dissociation constant for terbium binding to this repeat. Gadolinium also bound to CR3 and CR8, but caused a saturable decrease in tryptophan fluorescence in each case, rather than an increase (again similar to the behavior of terbium binding to these two repeats), that could be reversed by addition of calcium. The smaller magnitude of the effect did not permit accurate determination of the dissociation constant for Gd^{3+} binding to CR3 or CR8. These results again show that all domains have a common calcium site that can also bind lanthanides, but that there are basic differences in the position of the indole ring relative to the metal ion between LB1 and the two repeats from LRP.

HSQC Spectra of CR3 and CR8. [^1H-^{15}N]-HSQC spectra of CR3 and CR8 in the absence of calcium showed poor spectral dispersion in the proton dimension and only about half of the expected amide correlations, whereas spectra of

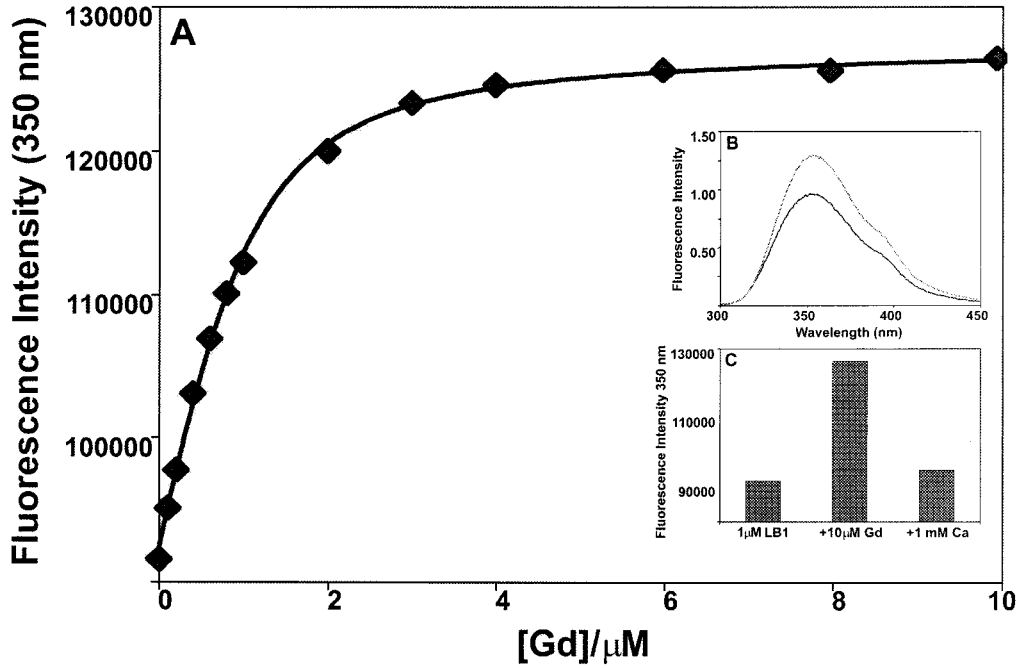

FIGURE 6: Gadolinium binding to 1 μM LB1 at pH 7.4 in 20 mM HEPES, monitored by the change in tryptophan fluorescence. The solid line is the fit to the experimental data. The two insets show (top) tryptophan emission spectra in the absence (solid line) and presence of 10 μM $GdCl_3$ (shaded line) and (bottom) the fluorescence intensity of 1 μM apo-LB1 and after successive additions of 10 μM $GdCl_3$ and 1 mM $CaCl_2$.

the calcium complexes showed very good dispersion in this dimension and all of the expected amide correlations (Figure 7). The poor dispersion and small number of correlations in the absence of calcium suggest that the protein can adopt multiple interconverting conformations in the absence of calcium with rapid exchange with solvent for many of the backbone amides, whereas the much greater dispersion and presence of all amide cross-peaks in the presence of calcium are consistent with calcium serving to lock the protein into a single rigid conformation.

As part of our study to determine the solution structure of CR3 and CR8, we are in the process of making all backbone and side chain NMR resonance assignments of these domains. Although these structure determinations do not form part of the present study, some chemical shift information from these determinations is relevant to the present study of the calcium coordination site. From the crystal structure, it is known that the calcium is coordinated to the carbonyls of the conserved tryptophan and of glycine in the sequence DGxxD in LB5 (coordinating glycine underlined). If these two carbonyls also coordinate to calcium in CR3 and CR8, it would be expected that the amide ^{15}N chemical shifts of the next (C-terminal) residue would be downfield-shifted by 4−8 ppm by such coordination, as has been well documented for other calcium binding proteins (*24*), and that the carbonyl carbons of the tryptophan and glycine residues themselves would be likewise downfield-shifted. We have ^{15}N assignments[2] for all of the backbone amides, as well as ^{13}C assignments for the carbonyls[2] of the tryptophan and glycine residues that are reported to coordinate to calcium in LB5. In CR3 the ^{15}N chemical shift of the aspartate C-terminal to the glycine has an ^{15}N chemical shift of 118 ppm compared with an expected range of 120−125 ppm, thus showing no indication of downfield perturbation by calcium coordination. The ^{13}C carbonyl chemical shift of the glycine is also normal, being 173.3 ppm compared with the expected random coil value of 173.6 ± 0.5 ppm (*25*). There were similar findings for the glycine in CR8 for which the ^{15}N chemical shift of the aspartate C-terminal to the glycine was again 118 ppm and the carbonyl ^{13}C chemical shift of the glycine was 173.1 ppm. In contrast, ^{15}N and ^{13}C chemical shift information, taken together, suggests that the tryptophan carbonyl probably remains a ligand to the metal ion in both CR3 and CR8. The ^{13}C carbonyl chemical shifts of the tryptophan in CR3 and CR8 are strongly downfield-shifted, 177.5 and 177.9 ppm, respectively, compared with the expected random coil value of 175.6 ± 0.5 ppm (*25*). The ^{15}N chemical shift data are less definitive, giving a chemical shift of 129 ppm for lysine adjacent to tryptophan in CR3, compared with an expected range of 124−129 ppm (*26*, *27*) and of 127 ppm for arginine adjacent to tryptophan in CR8 compared with an expected range of 125−129 ppm. These observations suggest that, whereas the tryptophan carbonyl remains a ligand to the calcium in both CR3 and CR8, the glycine carbonyl is not a calcium ligand in either of the two domains. CR3 and CR8 therefore have different metal ion coordination than LB5.

pH Dependence of Calcium Binding. LRP and other receptors in the LDL receptor family cycle in vivo between a neutral pH environment when on the cell surface and a low-pH (~5) environment when endocytosed and fused with endosomes. This pH cycle is thought to be the mechanism for taking up ligands outside the cell and releasing them inside as a result of a pH-dependent reduction in binding at the lower pH (*28*). It has furthermore been suggested that change in the calcium affinity, resulting from protonation of coordinating carboxyl groups, is the basis for this pH dependence of ligand binding (*28*). We therefore examined

[2] K. Dolmer, W. Huang, and P. G. W. Gettins, manuscript in preparation.

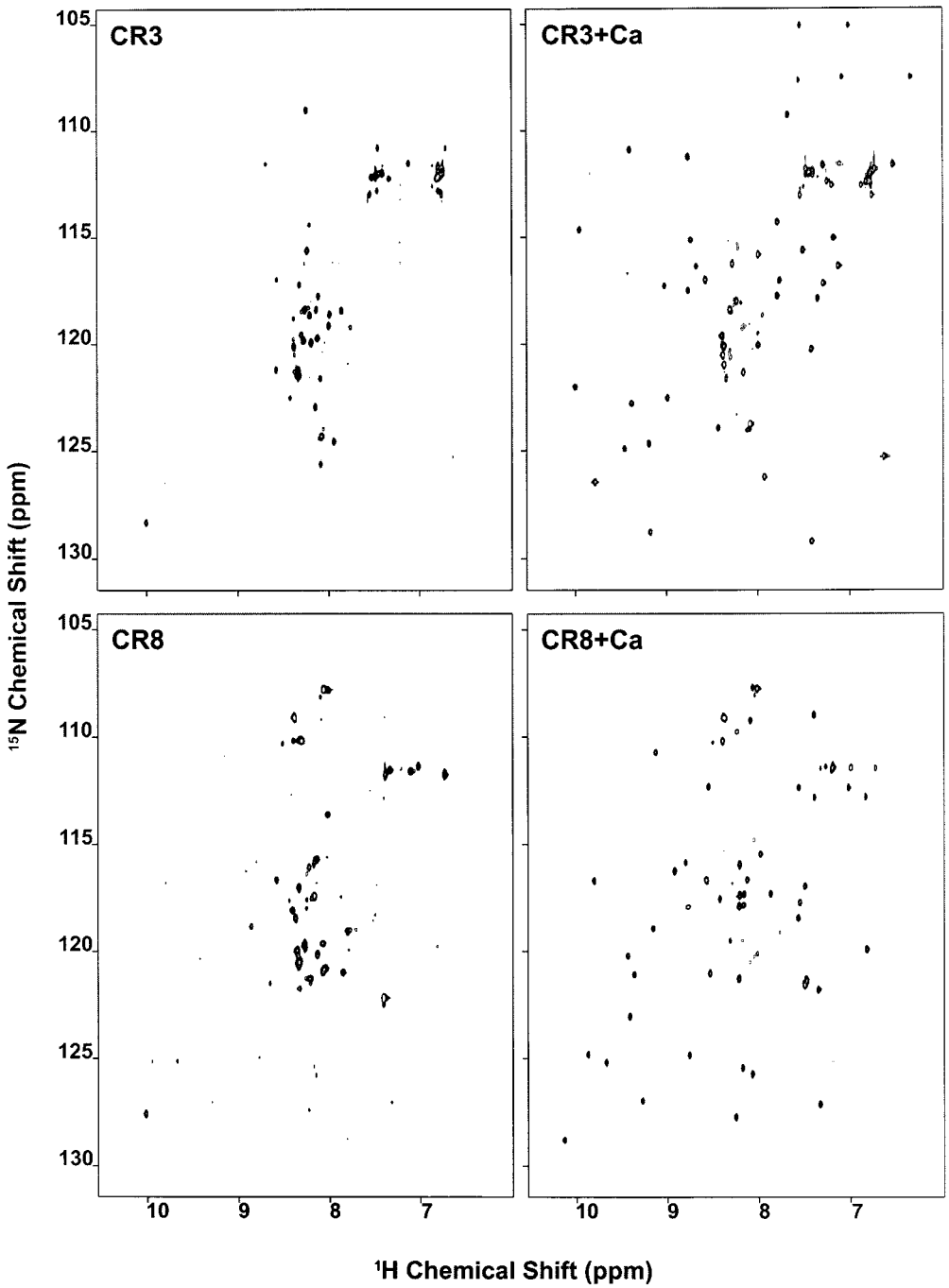

FIGURE 7: [^{1}H-^{15}N]-HSQC spectra of CR3 and CR8 in the absence and presence of calcium, showing the large increase in the detectable cross-peaks and in spectral dispersion upon calcium binding. The pH was 5.5. Samples with calcium had 10 mM $CaCl_2$ added to ensure saturation at this pH.

the pH dependence of calcium binding to CR3 and CR8 using change in tryptophan fluorescence. CR8 showed a very strong decrease in binding affinity between pH 6 and pH 5 (Figure 8B), with a shift in the midpoint of the titration by a factor of 100 in calcium concentration. The effect for CR3 was less dramatic (Figure 8A), with a more gradual and smaller reduction in affinity, such that the site was still about 50% occupied at 1 mM calcium and pH 4.5. If calcium-induced structural changes are indeed used in vivo to switch the complement repeats from high to low ligand affinity, our present findings on the pH dependence of calcium affinity suggest that individual repeats will show markedly different response to lowering of pH and thus to release of protein ligand.

DISCUSSION

We have shown from perturbation of tryptophan fluorescence that two complement-like repeats from LRP, CR3 and CR8, possess a tight calcium binding site that appears to be homologous in location to the calcium site present in repeats from the LDL receptor: LB1, LB2, and LB5. The dissociation constants were similar to those of other calcium binding proteins (*29*) and are tight enough that 100−200 μM calcium was sufficient to saturate the site. Lanthanides could also bind at this site and did so with even higher affinity, resulting from the higher charge compared with calcium. Thus, gadolinium caused saturable changes in tryptophan fluorescence for all three repeats examined here, which could be

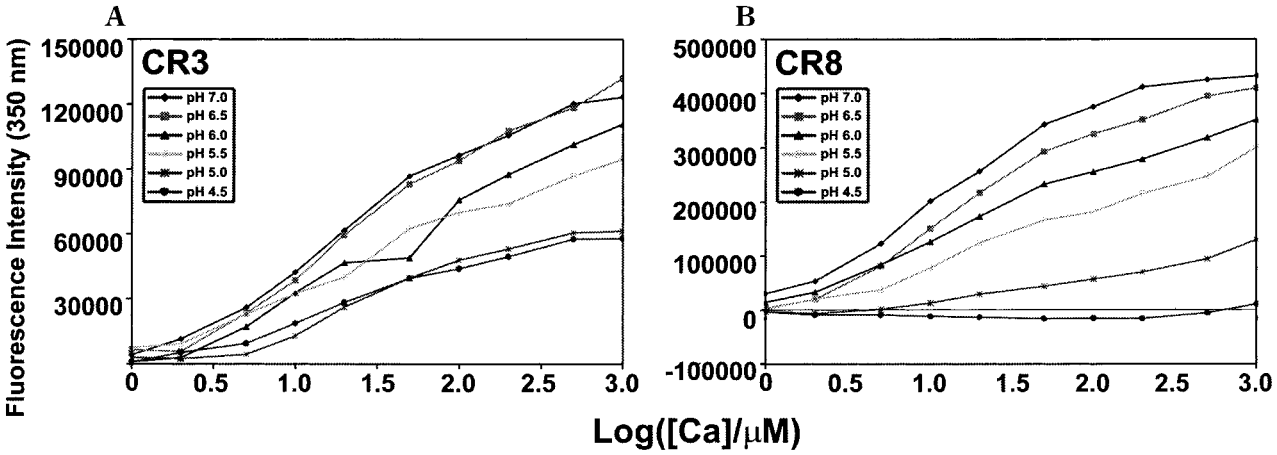

FIGURE 8: pH dependence of calcium binding to CR3 and CR8, determined from the change in tryptophan fluorescence. (A, left panel) CR3; (B, right panel) CR8. Titrations were carried out at pH 7.0, 6.5, 6.0, 5.5, 5.0, and 4.5.

reversed by addition of excess calcium. Terbium gave a saturable increase in sensitized terbium fluorescence, which could also be reversed by added calcium. The occurrence of sensitized terbium fluorescence showed that this calcium binding site must be very close to the single tryptophan that is present in all three repeats, since such sensitized fluorescence depends on Förster resonance energy transfer to the terbium and therefore requires close proximity between the terbium acceptor and the donor tryptophan for the mechanism to be very efficient (*30*). Calcium binding also caused major changes in the [^{1}H-^{15}N]-HSQC spectra of CR3 and CR8, consistent with a change from a conformationally flexible structure to a rigid domain, as indicated by the backbone amide signals, and a local perturbation of the tryptophan indole side chain, as indicated by chemical shift perturbation of the indole amide signal. CR3 and CR8 showed pH dependent binding of calcium in the pH range 4−7, as expected from the likely coordination by carboxyl ligands. However, the steepness of the pH dependence was very much more marked for CR8 than CR3, suggesting that each complement-like repeat has a unique metal binding site.

It has been shown that four carboxyl groups are among the ligands to the calcium ion in LB5 (*16*). It is therefore likely that any of the complement-like repeats that possesses these residues will also possess a calcium binding site and that calcium will induce a pH-dependent rigidification of the structure. Of the 31 such repeats in LRP, 23 have all 4 of these residues conserved, including CR3 and CR8. In LDLR, six of the seven repeats contain these residues, including LB1, LB2, and LB5. Despite the likelihood that at least 23 of the complement-like repeats of LRP and 6 of the 7 repeats of LDLR contain a calcium binding site, it is not expected, based on results from the present study, that all of these calcium binding sites will be equivalent. In comparing the behavior of the three repeats examined here, it seems that there is a fundamental difference between the calcium binding sites in CR3 and CR8 compared with the site in LB1. The latter gave only a small enhancement in tryptophan fluorescence upon binding calcium and a very large enhancement upon binding gadolinium. Conversely, CR3 and CR8 gave large enhancements in tryptophan fluorescence upon binding calcium, but decreases upon binding gadolinium. CR3 and CR8 also gave more modest increases in terbium fluorescence compared with LB1. This suggests some difference in position of the metal binding site with respect to the tryptophan side chain in LB1 compared with CR3 and CR8. Although ^{15}N and ^{13}C chemical shift data for backbone amide and carbonyls suggest that the tryptophan carbonyl remains a calcium ligand in CR3 and CR8, these data strongly suggest that the glycine four residues C-terminal is no longer a ligand in either domain.

Such a difference in metal ion coordination is likely to result from the differences in primary structure of CR3 and CR8 compared with LB1. In the sequence CDxxxDC (Figure 1), where the two aspartic acid residues are two of the four conserved calcium ligands found in LB5, CR3 and CR8 have an additional aspartate as the middle "x" residue. The crystal structure of LB5 shows that this residue is well placed to coordinate to the metal ion if it were an aspartate or glutamate. Thus, in CR3 and CR8 there are five carboxyl groups available to coordinate the calcium, whereas there are only four in LB1, LB2, and LB5. This might result in preferential coordination to the extra carboxyl in CR3 and CR8 and a consequent shift in the position of the metal ion, accounting for the observed differences in the fluorescence perturbations, together with loss of the glycine carbonyl as a ligand. Indeed, since the extra aspartate is adjacent to the glycine, which is adjacent to another calcium binding aspartate, it would be conformationally difficult for the two carboxyl groups of the aspartates in the sequence CDGD (aspartates underlined) to coordinate the metal ion at the same time as the carbonyl of the intervening glycine. It is possible that this difference in metal coordination may be of critical importance in determining ligand specificity. LDLR has a much more restricted ligand specificity than does LRP, and none of the complement-like repeats of LDLR has these five potential calcium ligands. In contrast, 19 of the 31 repeats of LRP have these 5 carboxyl residues. Furthermore, the distribution within LRP is not even; 7 out of 8 repeats in cluster II, and 8 out of 11 in cluster IV, contain all 5 carboxyls, whereas only 4 out of 10 repeats in cluster III contain them. It has been shown that cluster II is a principal binding site for ligands that bind to LRP but not to LDLR, such as α_2-macroglobulin−proteinase complexes and certain serpin−proteinase complexes. There may therefore be a functional connection between such a pentacoordinate calcium site and specificity for certain types of protein ligand.

Even for complement-like repeats within LRP that possess five potential carboxyl ligands to the calcium site, there are differences in properties that have been identified here for CR3 and CR8. Thus, the fluorescence intensity of the single tryptophan is about 70% higher in CR8 than in CR3 and shows a greater increase, both absolute and in percentage terms, upon calcium binding. Of more functional significance, the calcium site in CR8 shows a much steeper pH dependence, such that the calcium site is nearly unoccupied at the pH of the endosome, whereas the site in CR3 is still about 50% occupied at 100 μM calcium.

The present finding that both gadolinium and terbium bind both to complement repeats from LRP and to LB1 from LDLR is at variance with an earlier study that reported the failure of gadolinium to bind specifically to LB1 (*23*). It is possible that the difference results from the sample in the latter study not being metal-free prior to addition of gadolinium. Those authors added EDTA to LB1 to create the metal-free state and high calcium to create the calcium-bound state. However, for the gadolinium titration, it was presumably necessary to add gadolinium to a metal-free sample in the absence of EDTA. Because of the high affinity of these complement-like repeats for metal ions, great care must be taken to ensure that metal-free preparations remain in the apo state.

REFERENCES

1. Moestrup, S. K. (1994) *Biochim. Biophys. Acta Rev. Biomembr. 1197*, 197−213.
2. Herz, J., Kowal, R. C., Goldstein, J. L., and Brown, M. S. (1990) *EMBO J. 9*, 1769−1776.
3. Poller, W., Willnow, T. E., Hilpert, J., and Herz, J. (1995) *J. Biol. Chem. 270*, 2841−2845.
4. Storm, D., Herz, J., Trinder, P., and Loos, M. (1997) *J. Biol. Chem. 272*, 31043−31050.
5. Kasza, A., Petersen, H. H., Heegaard, C. W., Oka, K., Christensen, A., Dubin, A., Chan, L., and Andreasen, P. A. (1997) *Eur. J. Biochem. 248*, 270−281.
6. Moestrup, S. K., and Gliemann, J. (1989) *J. Biol. Chem. 264*, 15574−15577.
7. Kounnas, M. Z., Moir, R. D., Rebeck, G. W., Bush, A. I., Argraves, W. S., Tanzi, R. E., Hyman, B. T., and Strickland, D. K. (1995) *Cell 82*, 331−340.
8. Willnow, T. E., Goldstein, J. L., Orth, K., Brown, M. S., and Herz, J. (1992) *J. Biol. Chem. 267*, 26172−26180.
9. Zdanovsky, A. G., Chiron, M., Pastan, I., and FitzGerald, D. J. (1993) *J. Biol. Chem. 268*, 21791−21799.
10. Williams, S. E., Ashcom, J. D., Argraves, W. S., and Strickland, D. K. (1992) *J. Biol. Chem. 267*, 9035−9040.
11. Nielsen, M. S., Nykjaer, A., Warshawsky, I., Schwartz, A. L., and Gliemann, J. (1995) *J. Biol. Chem. 270*, 23713−23719.
12. Horn, I. R., Van den Berg, B. M. M., Van der Meijden, P. Z., Pannekoek, H., and van Zonneveld, A. J. (1997) *J. Biol. Chem. 272*, 13608−13613.
13. Avramoglu, R. K., Nimpf, J., McLeod, R. S., Ko, K. W. S., Wang, Y. W., FitzGerald, D., and Yao, Z. M. (1998) *J. Biol. Chem. 273*, 6057−6065.
14. Daly, N. L., Scanlon, M. J., Djordjevic, J. T., Kroon, P. A., and Smith, R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6334−6338.
15. Daly, N. L., Djordjevic, J. T., Kroon, P. A., and Smith, R. (1995) *Biochemistry 34*, 14474−14481.
16. Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997) *Nature 388*, 691−693.
17. Moestrup, S. K., Kaltoft, K., Sottrup-Jensen, L., and Gliemann, J. (1990) *J. Biol. Chem. 265*, 12623−12628.
18. Smith, D. B., and Johnson, K. S. (1988) *Gene 67*, 31−40.
19. Blacklow, S. C., and Kim, P. S. (1996) *Nat. Struct. Biol. 3*, 758−761.
20. Fesik, S. W., and Zuiderweg, E. R. P. (1990) *Q. Rev. Biophys. 23*, 97−131.
21. Muhandiram, D. R., and Kay, L. E. (1994) *J. Magn. Reson. Ser B. 103*, 203−216.
22. Palmer, A. G., III, Cavanagh, J., Wright, P. E., and Rance, M. (1994) *J. Magn. Reson. 93*, 151−170.
23. Atkins, A. R., Brereton, I. M., Kroon, P. A., Lee, H. T., and Smith, R. (1998) *Biochemistry 37*, 1662−1670.
24. Biekofsky, R. R., Martin, S. R., Browne, J. P., Bayley, P. M., and Feeney, J. (1998) *Biochemistry 37*, 7617−7629.
25. Wishart, D. S., and Sykes, B. D. (1994) *Methods Enzymol. 239*, 363−392.
26. Gluschka, M., Lee, M., Coffin, S., and Cowburn, D. (1990) *J. Am. Chem. Soc. 112*, 2843.
27. Braun, D., Wider, G., and Wüthrich, K. (1994) *J. Am. Chem. Soc. 116*, 8466−8469.
28. Brown, M. S., Herz, J., and Goldstein, J. L. (1997) *Nature 388*, 629−630.
29. Gilli, R., Lafitte, D., Lopez, C., Kilhoffer, M. C., Makarov, A., Briand, C., and Haiech, J. (1998) *Biochemistry 37*, 5450−5456.
30. Horrocks, W. DeW., Jr. (1993) *Methods Enzymol. 226*, 495−538.

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