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A novel chemical footprinting approach identifies critical lysine residues involved in the binding of receptor-associated protein to cluster II of LDL receptor-related protein

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Tandem mass tags (TMTs) were utilized in a novel chemical footprinting approach to identify lysine residues that mediate the interaction of receptor-associated protein (RAP) with cluster II of LDL (low-density lipoprotein) receptor (LDLR)-related protein (LRP). The isolated RAP D3 domain was modified with TMT-126 and the D3 domain–cluster II complex with TMT-127. Nano-LC–MS analysis revealed reduced modification with TMT-127 of peptides including Lys^{256}, Lys^{270} and Lys^{305}-Lys^{306} suggesting that these residues contribute to cluster II binding. This agrees with previous findings that Lys^{256} and Lys^{270} are critical for binding cluster II sub-domains [Fisher, Beglova and Blacklow (2006) Mol. Cell **22**, 277–283]. Cluster II-binding studies utilizing D3 domain variants $K^{256}A$, $K^{305}A$ and $K^{306}A$ now showed that Lys^{306} contributes to cluster II binding as well. For full-length RAP, we observed that peptides including Lys^{60}, Lys^{191}, Lys^{256}, Lys^{270} and Lys^{305}-Lys^{306} exhibited reduced modification with TMT in the RAP–cluster II complex. Notably, Lys^{60} has previously been implicated to mediate D1 domain interaction with cluster II. Our results suggest that also Lys^{191} of the D2 domain contributes to cluster II binding. Binding studies employing the RAP variants $K^{191}A$, $K^{256}A$, $K^{305}A$ and $K^{306}A$, however, revealed a modest reduction in cluster II binding for the $K^{256}A$ variant only. This suggests that the other lysine residues can compensate for the absence of a single lysine residue for effective complex assembly. Collectively, novel insight has been obtained into the contribution of lysine residues of RAP to cluster II binding. In addition, we propose that TMTs can be utilized to identify lysine residues critical for protein complex formation.

Key words: footprinting, low-density lipoprotein (LDL) receptor-related protein, ligand binding, mass spectrometry, receptor-associated protein, tandem mass tag.

INTRODUCTION

The assembly of a protein complex is a key event in almost all biological processes. To gain insight into these mechanisms and defects therein, it is of critical importance to understand how proteins interact. Yet, identification of protein interaction sites has remained a continuous challenge [1]. An evolving technology for the identification of interactive regions on proteins involves chemical modification of amino acid regions of unbound and bound proteins [2–4]. The amino acid residues that contribute to complex assembly are expected to be protected from chemical modification. In 2009, Ori et al. [5] demonstrated in an elegant study that heparin-binding sites can be identified employing a selective labelling strategy.

A major issue to overcome using a chemical footprinting approach is that the equilibrium between bound and unbound states of proteins will also allow for unintended chemical modification of residues that are critical for the interaction. Especially for proteins that bind with low affinity, the specific protection from chemical modification may be hardly, or not at all, observed. To overcome this issue, footprinting approaches have been developed that rely on fast modification of protein complexes. These methods include, for instance, hydroxyl radical oxidation of amino acid regions of a protein complex [3].

Previously, we and others employed lysine-directed isobaric tandem mass tags (TMTs) to assist in the structural characterization of proteins using MS [6–8]. In the present study, we explore the potential of these mass tags to effectively identify lysine residues that directly contribute to protein complex formation. In the employed approach, we make use of the isobaric TMTs TMT-126 and TMT-127. Modification of the lysine residues of assembled proteins with TMT-127 and the unbound proteins with TMT-126 is expected to lead to a reduced incorporation with TMT-127 in amino acid regions comprising lysine residues that contribute to complex formation. Taking maximum advantage of the fact that TMT-126 and TMT-127 exhibit a different isotope distribution, MS/MS fragmentation of modified peptides derived from these regions allows for relative quantification of the incorporation with TMT-126 and TMT-127 [9]. As a model system, we employ the complex between receptor-associated-protein (RAP) and cluster II of the ligand-binding domains of low-density lipoprotein (LDL) receptor (LDLR)-related protein (LRP). This complex is particularly suitable for the

Abbreviations: CID, collision-induced dissociation; HCD, higher energy collision-induced dissociation; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LRP, low-density lipoprotein receptor-related protein; RAP, receptor-associated protein; TMT, tandem mass tag; WT, wild-type.

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present study as (i) lysine residues have been shown to contribute to complex formation [10–12], (ii) the identity of three critical lysine residues of RAP has been established from crystallography and NMR studies [10,13], and (iii) the contribution of additional lysine residues remains to be assessed.

LRP is a member of the LDLR family and has been implicated to play a role in a range of biological processes including cell migration, vascular permeability and the catabolism of coagulation proteins [14]. The physiological role of RAP is to assist in the proper intracellular folding of the LDLR family members and to prevent premature intracellular ligand binding [15]. Because of the latter characteristic, RAP has been frequently employed as an antagonist to identify novel binding partners of LRP [16]. In addition, the complex between RAP and ligand-binding domains has been studied to gain insight into the general mechanism of complex assembly between LRP and its ligands [11–13,15–20].

RAP comprises three similar D domains, each of which has been demonstrated to bind LDLR and LRP. Although there is a debate in literature about the actual binding affinities of the individual domains, it has been proposed that the isolated D3 domain binds more effectively to the LRP–LDLR ligand-binding domains than the isolated D1 and D2 domains [18,20]. The ligand-binding domains of the LDLR-like proteins are, in turn, small compact domains that are clustered in distinct regions within the protein. LDLR comprises a single cluster containing seven of these so-called complement type repeats, whereas LRP contains four of these specialized ligand-binding regions [21].

Crystal structure analysis of the RAP D3 domain in complex with two repeats from LDLR has revealed that Lys^{256} and Lys^{270} of the D3 domain are critical for the interaction [10]. The structure shows that each of these lysine residues is inserted into an 'acidic necklace' of negatively charged residues of a single complement-type repeat [10,17]. We and others have demonstrated that an arginine residue cannot replace the lysine residue in this binding mechanism [10,12,13,22]. NMR analysis of the RAP D1 domain in interaction with two complement-type repeats from LRP revealed that Lys^{60} is critical for the interaction with a complement-type repeat [13]. No information is available about the lysine residues of the D2 domain that may interact with an acidic necklace of a ligand-binding domain of LRP.

Using our footprinting approach, we confirm that Lys^{60}, Lys^{256} and Lys^{270} contribute to the binding of RAP to LRP cluster II. Our results together further suggest that lysine residues at position 191 of the D2 domain and position 306 of the D3 domain are involved in LRP cluster II binding as well.

EXPERIMENTAL

Proteins

Human RAP D3 domain was purified from *Escherichia coli* DH5α cells as described in [12,19]. Full-length rat GST–RAP was expressed and purified as described in [12,16]. Variants of human RAP D3 domain and full-length rat RAP were constructed using Quik Change mutagenesis (Stratagene) according to the instructions of the manufacturer using appropriate primers. LRP1 cluster II was expressed in baby hamster kidney (BHK) cells and purified as described in [23]. This cluster II fragment contains an amino acid tag which is utilized for detection of cluster II with horseradish peroxidase-labelled monoclonal antibody CLB–CAg69 [24,25]

TMT modification

Human RAP D3 domain or full-length RAP was incubated in presence or absence of a 1- or 10-fold molar excess of LRP cluster II for 15 min at 37 °C in 50 mM HEPES, pH 7.4, 150 mM NaCl, and 5 mM $CaCl_2$. RAP D3 domain or full-length RAP (2 μg in total) were incubated with a 10 000-fold molar excess of TMT-126 and the RAP–cluster II complex with TMT-127 for 15 min at 37 °C. The TMT-labelling reaction was terminated by the addition of 150-fold molar excess of hydroxylamine over the TMTs. Protein mixtures were pooled at a 1:1 ratio and the cysteines were alkylated as described in [6]. Proteins were proteolysed by either chymotrypsin or Glu-C or Asp-N according to the instructions of the manufacturer (Thermo Fisher Scientific). Obtained peptides were desalted employing a C_{18} ZipTip (Millipore Corporation) according to the instructions of the manufacturer.

MS analysis

Peptides were separated by reverse-phase chromatography and sprayed into a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) essentially as described in [6,26]. During reverse-phase chromatography, we utilized a 40-min gradient from 0 % to 35 % (v/v) acetonitrile with 0.5 % (v/v) acetic acid. Collision-induced dissociation (CID) spectra and higher energy CID (HCD) spectra were acquired as described in Dayon et al. [9]. The three most intense precursor ions in the full scan (300–2000 *m/z*, resolving power 30 000) with a charge state of 2 + or higher were selected for CID using an isolation width of 2 Da, a 35 % normalized collision energy and an activation time of 30 ms. The same precursor ions were subjected to HCD with a normalized collision energy of 60 %, which allows for the identification of the reporter group from the TMT label.

Identification of the peptides as well as the TMT-127/TMT-126 ratio thereof

The identification of the peptides and determination of their TMT-127/TMT-126 ratio were assessed employing Proteome Discoverer software 1.2. The SEQUEST search algorithm was used employing the protein database 25.H_sapiens.fasta including the amino acid sequence of human RAP D3 domain or a database containing RAP from the rat. The following selection criteria were used: (i) all lysine residues are modified by a TMT label, (ii) all cysteine residues are alkylated, (iii) all methionine residues may be oxidized, and (iv) a maximum false discovery rate of 5 % was accepted. The TMT ratio of the identified peptides was normalized to the average TMT ratio obtained within that experiment. We also verified whether the labelling of RAP with TMT-127 and the RAP–cluster II complex with TMT-126 affects the outcome of the experiments (TMT labels are reversed in this experiment). This was, however, not the case (Supplementary Figure S1).

Solid-phase competition assay

Next 1 μg/ml wild-type (WT) human RAP D3 domain or 0.5 μg/ml full-length WT rat RAP was immobilized at 4 °C overnight on a microtitre plate in 0.05 M $NaHCO_3$, pH 9.8. Plates were washed with TBS containing 5 mM $CaCl_2$ and 0.1 % Tween 20. LRP cluster II (2.5 nM) was incubated with RAP D3 domain and 0.5 nM cluster II with full-length RAP for 2 h at 37 °C in the presence of increasing concentrations of RAP D3 domain variants or full-length RAP variants. Residual cluster II binding to immobilized RAP D3 domain and full-length RAP was detected

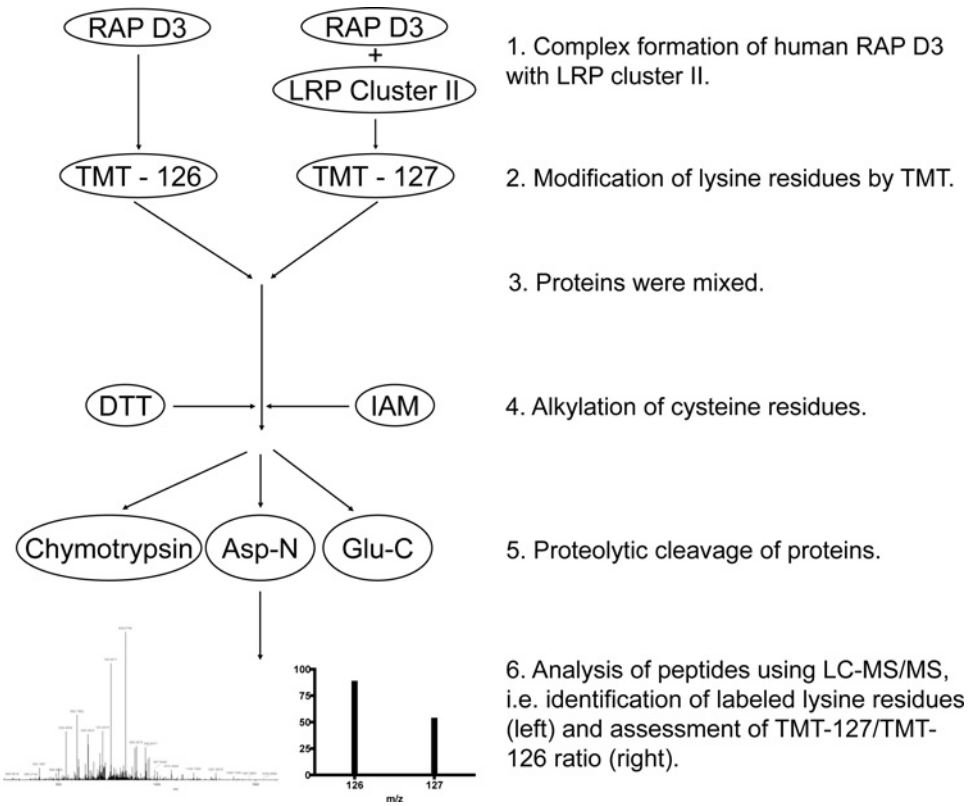

Figure 1 Work flow of the chemical footprinting approach

The isolated RAP D3 domain was modified with TMT-126 in the absence of cluster II and with TMT-127 in the presence of cluster II. Proteins were pooled in a 1:1 molar ratio and proteolysed by chymotrypsin, Asp-N or Glu-C. Peptides were subsequently analysed on a nano-LC Orbitrap XL mass spectrometer.

employing horseradish peroxidase-labelled monoclonal antibody Cag69 as described in [24,25].

RESULTS

Work flow of the chemical footprinting based MS approach using the RAP D3 domain–LRP cluster II complex as model

As crystal structure analysis and mutagenesis studies have demonstrated that the lysine residues at positions 256 and 270 of the D3 domain of RAP bind directly to two complement-type repeats [10–12], we employed this domain to validate our approach. To this end, the lysine residues of the D3 domain were modified with an excess of TMT-126 in the absence of LRP cluster II and with TMT-127 in the presence of an excess of LRP cluster II. The modification of the lysine residues was allowed for 15 min at 37 °C and the reaction was stopped with hydroxylamine. The proteins were subsequently pooled in an equal molar ratio based on the concentration of RAP. Pooled proteins were alkylated and divided into three fractions to enable proteolysis by chymotrypsin, Asp-N and Glu-C. The resulting peptides were analysed employing a nano-LC LTQ Orbitrap XL mass spectrometer. CID of the peptide ions was employed to identify the peptides. HCD fragmentation of the same peptide was utilized to detect the reporter groups from TMT-126 and TMT-127 [9]. Protection of a lysine residue from chemical modification in the presence of LRP cluster II is expected to result in a decreased TMT-127/TMT-126 ratio. A ratio of 1 indicates that the exposure of the involved lysine residue to the solvent is not altered upon complex formation. Figure 1 shows a schematic overview of the work flow.

Identification of lysine residues of the RAP D3 domain that contribute to receptor binding

The peptide mixtures obtained from the above-described approach were analysed by MS. The three most abundant peptide ions between 300 and 2000 *m/z* in each full scan were subjected to CID and HCD. In total, 23 unique peptides of the D3 domain were identified covering 88 % of the complete sequence and all lysine residues of this domain (Supplementary Table S1). Figure 2 shows part of the HCD spectra of the TMT-modified peptides ^{251}EAKIEKHNHY^{260}, ^{256}KHNHYQKQLE^{265} and ^{266}IAHEKLRHAE^{275}. The average TMT-127/TMT-126 ratio obtained from at least four independent experiments is displayed in Figure 3. The data revealed a decrease in the ratio for the peptides including the lysine residues at positions 256 and/or 270. This suggests enhanced protection of these residues from modification by TMT-127 in the presence of cluster II implying that Lys^{256} and Lys^{270} contribute to the binding interaction. This is in full agreement with the crystal structure that shows that these residues contribute directly to the interaction with two consecutive complement-type repeats (Figure 3B) [10]. Intriguingly, we found that the peptides that include the lysine residues at positions 305 and 306 exhibit a marked decrease in TMT-127/TMT-126 ratio as well (Figure 3A). This suggests that either Lys^{305} or Lys^{306} or both may contribute to the direct interaction with a third complement

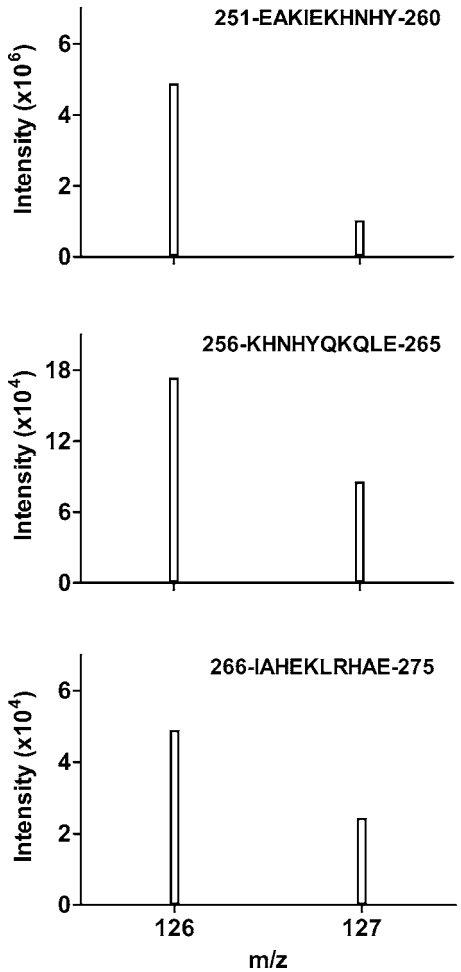

Figure 2 Peptides including Lys^{256} and Lys^{270} exhibit reduced incorporation with TMT-127

The RAP D3 domain was modified with TMT-126 in the absence of cluster II and with TMT-127 in the presence of cluster II. Proteins were mixed in a 1:1 molar ratio, cleaved into peptides and analysed by MS. CID and HCD spectra are obtained of the TMT-modified peptide ions derived from the RAP D3 domain as described in the Experimental section. Shown is part of HCD spectra that comprise the mass reported groups derived from TMT-126 and TMT-127 of the indicated peptides.

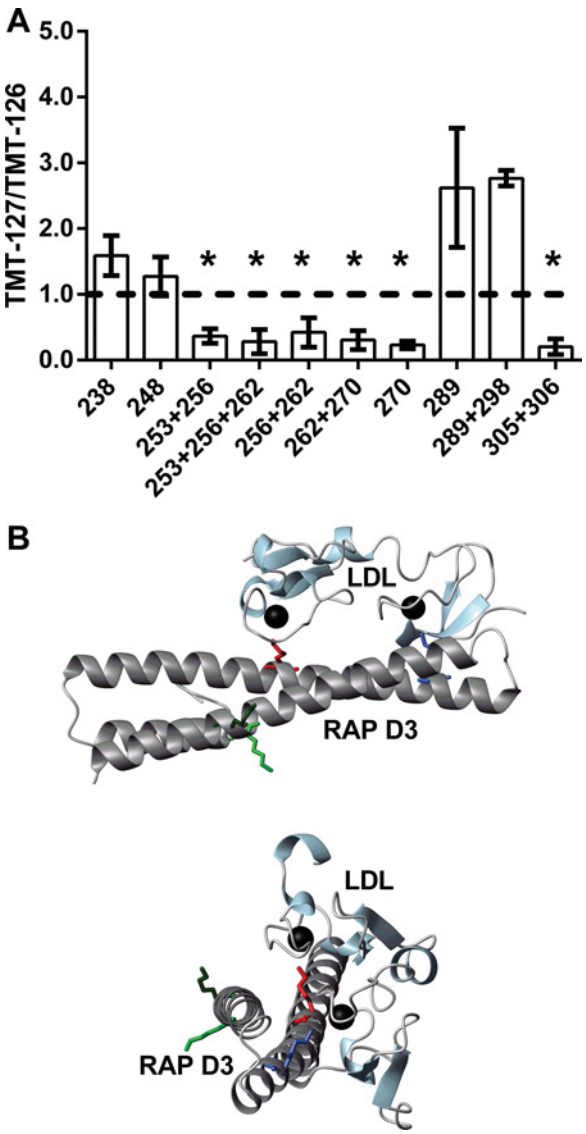

Figure 3 Average TMT-127/TMT-126 ratio of the identified lysine-containing peptides of the RAP D3 domain

(**A**) The RAP D3 domain was modified with TMT-126 in the absence of cluster II and with TMT-127 in the presence of cluster II. Based on the concentration of the D3 domains, proteins were mixed in a 1:1 molar ratio, cleaved into peptides and analysed by MS. The average TMT-127/TMT-126 ratio obtained from at least four independent experiments of peptides comprising the same lysine residues is displayed. Lysine residue positions are indicated on the *x*-axis, those by which the average TMT-127/TMT-126 ratio is more than two S.D.s below 1 are indicated with an asterisk. (**B**) Two orientations of the crystal structure of the RAP D3 domain (in grey) in complex with two complement-type repeats of LDLR (in blue) (PDB 2FCW) [10]. Indicated in red is Lys^{256} and in blue is Lys^{270}. Lys^{305} is shown in light green and Lys^{306} is shown in dark green. The black spheres are calcium atoms that are critical for the structural integrity of the repeats.

type repeat of LRP cluster II (Figure 3B). Remarkably, the peptides including the lysine residues at position 238 or 289 show a marked increase in the incorporation of TMT-127 implying that these residues have an increased surface exposure in the presence of cluster II. Taken together, next to Lys^{256} and Lys^{270} the lysine residues at positions 305 and/or 306 also mediate RAP D3 domain binding to LRP cluster II.

Identification of critical LRP-binding lysine residues within the D3 domain of full-length RAP

We next evaluated whether we can identify the critical lysine residues of the D3 domain employing full-length RAP of rat origin. Full-length RAP of human and rat origin share 75 % sequence identity and 87 % sequence similarity (Supplementary Figure S2) [27]. The RAP D3 domains share 81 % sequence identity and 92 % sequence similarity. All lysine residues of the human RAP D3 domain are conserved in rat RAP. Employing the footprinting approach, 94 peptides of full-length RAP were identified covering 89 % of the sequence of the protein. The TMT-modified peptides included 34 out of 37 lysine residues of RAP (Supplementary Table S2). The obtained average TMT-127/TMT-126 ratio of the lysine-containing peptides derived from the D3 domain is shown in Figure 4(C). The enhanced modification with TMT-127 of the lysine residues 238 and 289, which was observed for the isolated D3 domain in complex with cluster II (Figure 3A), is not found employing the full-length RAP–cluster II complex. However, the result does again reveal that Lys^{256} and Lys^{270} exhibit a reduced incorporation of TMT-127 (Figure 4C). In addition, the

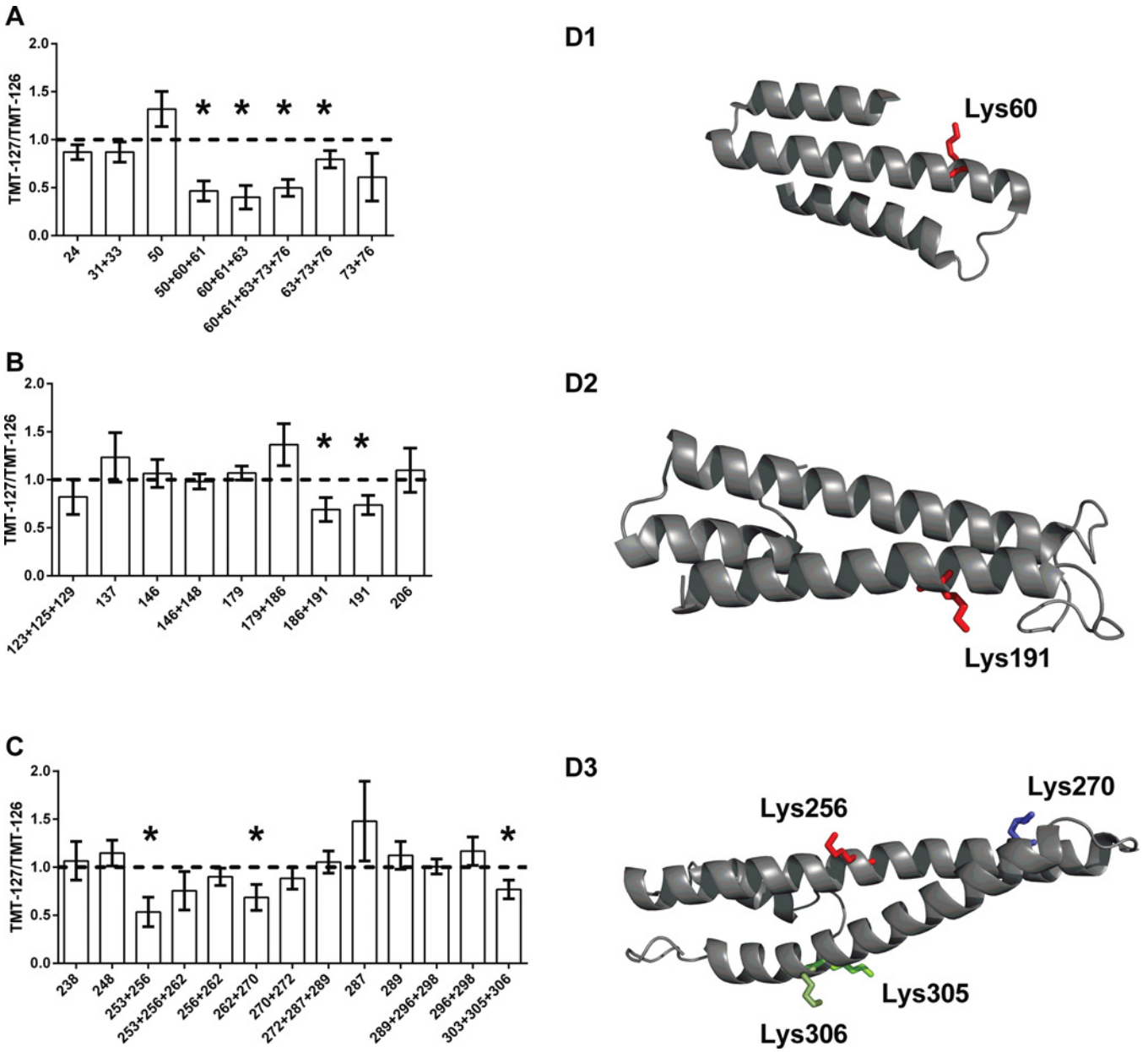

Figure 4 Average TMT-127/TMT-126 ratio of the identified lysine-containing peptides of full-length RAP

Full-length RAP was modified with TMT-126 in the absence of cluster II and with TMT-127 in the presence of cluster II. Proteins were mixed in a 1:1 molar ratio, cleaved into peptides and analysed by MS. The average TMT-127/TMT-126 ratio obtained from at least four independent experiments of peptides comprising the same lysine residues is displayed. Lysine residue positions are indicated on the *x*-axis. The top panel shows the lysine residues from the D1 domain of RAP, the middle panel shows the lysine residues from the D2 domain and the bottom panel shows the lysine residues from the D3 domain. Shown on the right are the NMR structures of the individual domains of RAP (PDB 2P03) [28]. (**A**) Lys^{60} is displayed in red. (**B**) Lys^{191} is displayed in red. (**C**) Lys^{256} is displayed in red, Lys^{270} in blue, and Lys^{305} and Lys^{306} in light and dark green respectively. Lysine residues of which the average TMT-127/TMT-126 ratio is more than two S.D.s below 1 are indicated with an asterisk.

peptide including Lys^{305} and Lys^{306} also shows a decreased TMT-127/TMT-126 ratio (Figure 4C). This finding demonstrates that the residues of the D3 domain that contribute to LRP binding can be identified by our approach in both the isolated human D3 domain as well as the full-length rat RAP.

Identification of novel LRP-binding sites within RAP

Lys^{60} of the human RAP D1 domain (Figure 4A, right panel) has been implicated to interact with a complement-type repeat of cluster II [13]. No information is available about the lysine residues of the D2 domain that contribute to cluster II binding. In complete agreement with the NMR study, Figure 4(A) reveals a reduced incorporation of TMT-127 for the peptides including Lys^{60} of full-length RAP from rat (Figure 4A). A small decrease in the TMT-127/TMT-126 ratio was also observed for the peptide including the lysine residues at positions 63, 73 and 76. According to the NMR structure of RAP, these residues are, however, in close proximity to Lys^{60} [28]. It seems therefore seems unlikely that these residues interact with a second complement-type repeat of cluster II. For the D2 domain, only the peptides including Lys^{191}

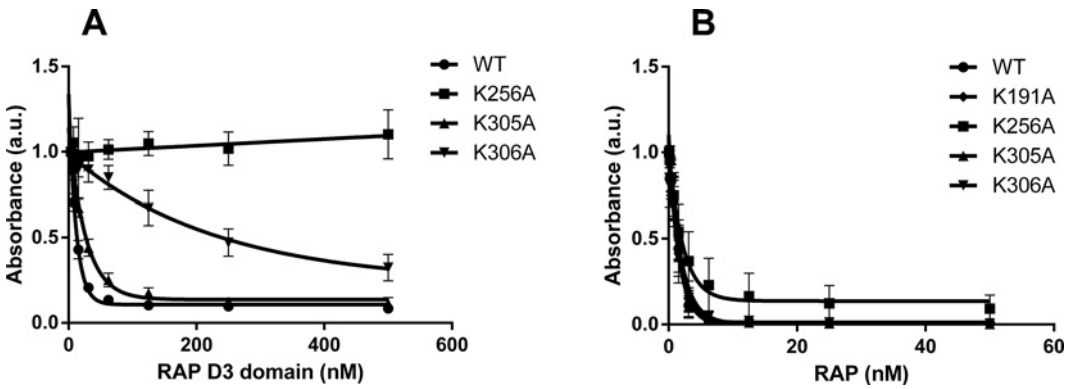

Figure 5 The role of the identified lysine residues for the interaction with cluster II

(**A**) Increasing concentrations of the indicated RAP D3 domain variants were incubated with 2.5 nM cluster II. The proteins were added to immobilized RAP D3 domain. Residual cluster II binding to immobilized D3 domain was assessed employing horseradish peroxidase-labelled monoclonal antibody CLB–CAg69 [24,25]. (**B**) Increasing concentrations of the indicated full-length RAP variants were incubated with 0.5 nM cluster II. The proteins were added to immobilized full-length RAP D3. Residual cluster II binding to immobilized D3 domain was assessed employing horseradish peroxidase-labelled monoclonal antibody CLB–CAg69.

showed a decreased TMT-127/TMT-126 ratio (Figure 4B). Our data strongly suggest a previously unidentified role of Lys^{191} for cluster II binding.

Lys^{306} contributes to the binding of the RAP D3 domain to cluster II

WT RAP and $K^{191}A$, $K^{256}A$, $K^{305}A$ and $K^{306}A$ variants were employed in a ligand-binding competition assay to evaluate the role of the identified residues for RAP–cluster II complex formation. To this end, WT RAP was immobilized to a microtitre plate and incubated with cluster II to assess the concentration at which half-maximum binding is reached (results not shown). This cluster II concentration (i.e. 0.5 nM) was then incubated with immobilized WT RAP in the presence of increasing concentrations of RAP and the variants thereof (Figure 5B). Results showed that all variants but one were equally effective in competing with immobilized RAP for binding cluster II. Only the $K^{256}A$ variant revealed a small decrease in the competition efficiency. Apparently, mutagenesis of a single contact site between RAP and cluster II has only a small effect on the binding interaction. We next assessed cluster II binding of the RAP D3 domain variants $K^{256}A$, $K^{305}A$ and $K^{306}A$ in a ligand-binding competition assay. Half-maximum binding of cluster II to the immobilized D3 domain was reached at a concentration of 2.5 nM. The competition data revealed that a concentration of 4 nM of WT D3 domain was required to obtain 50 % residual binding of cluster II to immobilized RAP D3 domain (Figure 5A). The D3 domain $K^{256}A$ variant was completely unable to inhibit the binding of cluster II to immobilized WT D3 domain. This agrees with the observation that Lys^{256} is critical for the interaction between WT D3 domain and a complement-type repeat. For $K^{305}A$ and $K^{306}A$ a concentration of 15 and 303 nM respectively was required to reach a residual binding of 50 %. This implies that mainly Lys^{306} contributes to LRP cluster II binding.

DISCUSSION

The crystal structure of the complex between RAP D3 domain and two-complement type repeats has increased our understanding about the mechanism of interaction between the RAP and the LDLR family members. The negatively charged residues of the two complement-type repeats form an acidic necklace around the positively charged side chains of the lysine residues at positions 256 and 270 [10]. The present study demonstrates that TMTs can be employed to successfully identify these lysine residues (Figures 2–4). Application of the approach also confirms the earlier established role of Lys^{60} for complex formation with a complement-type repeat. Our results together strongly suggest that the lysine residues, Lys^{191} and Lys^{306}, interact with the negatively charged acidic necklace of a complement-type repeat as well (Figures 3–5). We therefore propose that Lys^{60}, Lys^{191}, Lys^{256}, Lys^{270} and Lys^{306} are the critical lysine residues that directly contribute to complex formation between RAP and cluster II.

Chemical footprinting studies can be severely hampered by dissociation and re-association kinetics of a protein complex in solution. Critical residues can be modified upon dissociation of the complex, which may even lead to a conformational change of one of the binding partners. This will shift the binding equilibrium towards the unbound state of the proteins, which will further enhance TMT modification of the critical residues. It is therefore not surprising that the lysine residues that directly contribute to complex formation are also modified with TMT-127 in spite of the presence of cluster II (Figures 2–4). Furthermore, it can not be excluded that there is a dynamic equilibrium between distinct RAP–cluster II complexes in which one or more critical lysine residues are not occupied by a complement-type repeat. This may then explain why the difference in TMT ratios for full-length RAP are less pronounced than those obtained for the much smaller isolated D3 domain. In spite of these notions, the difference in chemical modification is sufficiently large to successfully identify the critical lysine residues.

In an elegant study by Dolmer et al. [29], the interaction between two complement-type repeats and Lys^{253}, Lys^{256}, Lys^{289} and Lys^{270} of the D3 domain was evaluated by replacing all other lysine residues with alanine. They showed that Lys^{253} and Lys^{289} support the interaction of the critical lysine pair at positions 256 and 270 with the two complement-type repeats. In our study, the intact LRP cluster II of nine complement-type repeats in interaction with the D3 domain revealed that Lys^{289} is more accessible for modification with TMT-127 in the protein complex. This implies that Lys^{289} is more exposed to the protein surface after complex formation. This suggests that Lys^{289} does not contribute to the direct interaction in the presence of multiple complement-type repeats. The increased TMT-127/TMT-126 ratio at Lys^{289} is not observed upon cluster II binding to full-length RAP from the

rat (Figure 4). This may be related to a difference in local structure of the D3 domain from rat and human origin. Alternatively, it cannot be excluded that Lys^{289} contributes to intra-domain interactions in full-length RAP. This interaction may remain unaltered upon binding cluster II. If so, this will then also not lead to an increased solvent exposure after cluster II binding.

The crystal structure as well as the NMR structure of RAP show that Lys^{306} is at the same side of the D3 domain as Lys^{256} and Lys^{270} (Figures 3B and 4C) [10,28]. The role of Lys^{306} for cluster II binding therefore raises, the possibility that three consecutive complement-type repeats of cluster II interact with the D3 domain of RAP. This is compatible with the observation that three complement-type repeats exhibit a higher binding affinity for the D3 domain than two complement-type repeats [20]. For the D1 domain of RAP, Lys^{60} has been identified to contribute to complex formation in the present (Figure 4A) and previous studies [13]. This suggests that a single complement-type repeat interacts with the D1 domain via the acidic necklace-binding mechanism. The positively charged residues Lys^{63}, Lys^{73} and Lys^{76} are in close proximity to Lys^{60} and may therefore support this interaction. This may explain the small reduction in TMT ratio of peptides that include Lys^{63}, Lys^{73} and Lys^{76}. The D2 domain also seems to interact with only a single complement-type repeat via the acidic necklace-binding model as only Lys^{191} showed a reduced TMT ratio. These findings are compatible with the previous observations that the isolated D1 and D2 domains are less effective in binding cluster II fragments [13,18].

The combined binding sites in RAP for the complement-type repeats mediate the particularly effective interaction with LRP. Our results suggest that even the absence of the critical residue Lys^{256} in full-length RAP can be compensated for by the other critical lysine residues (Figure 5). The notion that these binding sites are distributed over multiple domains of RAP provides an insight into the general mechanism by which LRP binds its ligand. The four clusters of complement type repeats in LRP may interact with a multitude of lysine residues that are distributed over a large area of the ligand. Pin-pointing a single binding site for LRP on a ligand may therefore not be possible. This provides, for instance, an explanation of why multiple LRP binding regions have been identified for coagulation factor VIII [23,30].

The chemical footprinting approach employed can be applied to any protein complex assembly that involves critical lysine residues. The interaction of CUB domains with protein-binding partners has, for instance, been suggested to involve lysine residues [31]. The identity of these lysine residues can now be reliably identified with the described approach. Taken together, we have developed a powerful approach to identify critical lysine residues for effective complex formation between proteins.

AUTHOR CONTRIBUTION

Esther Bloem and Eduard Ebberink performed the research and wrote the paper. Maartje van den Biggelaar contributed to the experimental design of the study and assisted in the purification of the required proteins. Carmen van der Zwaan provided technical assistance. Koen Mertens provided expert advice for the overall study and contributed to editing of the paper before submission. Alexander Meijer designed the research, provided guidance in the analysis and interpretation of the results and contributed to writing, drafting and pre-submission editing of the paper.

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