The Role of a Conserved Acidic Residue in Calcium-dependent Protein Folding for a Low Density Lipoprotein (LDL)-A Module

IMPLICATIONS IN STRUCTURE AND FUNCTION FOR THE LDL RECEPTOR SUPERFAMILY

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One common feature of the more than 1,000 complement-type repeats (or low density lipoprotein (LDL)-A modules) found in LDL receptor and the other members of the LDL receptor superfamily is a cluster of five highly conserved acidic residues in the C-terminal region, DXXXDXXXDXXDE. However, the role of the third conserved aspartate of these LDL-A modules in protein folding and ligand recognition has not been elucidated.

In this report, using a model LDL-A module and several experimental approaches, we demonstrate that this acidic residue, like the other four conserved acidic residues, is involved in calcium-dependent protein folding. These results suggest an alternative calcium coordination conformation for the LDL-A modules. The proposed model provides a plausible explanation for the conservation of this acidic residue among the LDL-A modules. Furthermore, the model can explain why mutations of this residue in human LDL receptor cause familial hypercholesterolemia.

The LDL receptor superfamily, with human LDL receptor as its prototype, consists of a large number of proteins such as the LDL receptor-related protein (LRP), gp330, and the very low density lipoprotein receptor (1). One common feature of these proteins is that they all contain at least one modular domain (called complement-type repeat, or LDL-A module) of ~40 residues in length, including six invariant cysteines and the C-terminal highly conserved acidic residue motif (DXXXXDXXXDXXDE). All together there are more than 1,000 known LDL-A modules found in a variety of proteins that are involved in diverse biological processes. In human LDL receptor, seven such imperfect repeats of LDL-A modules at the N terminus of the protein form the ligand binding domain, responsible for binding to its ligands, apoB and apoE (2–5). Naturally occurring point mutations in any of the conserved acidic residues of the individual LDL-A modules of human LDL receptor can cause familial hypercholesterolemia (FH), a genetic disease that ultimately leads to coronary heart disease and atherosclerosis (6). One proposed mechanism for these conserved acidic residues of LDL receptor in ligand binding is to interact with the basic residues of its ligands via ionic interactions (7–9).

Structural analysis of individual LDL-A modules, and recently the entire ectodomain of human LDL receptor, by x-ray crystallography revealed another mechanism by which these conserved acidic residues exert their role in protein conformation and function of LDL-A modules and thus LDL receptors (10–12). Among the five conserved acidic residues, DXXXXDXXXDXXDE, the side chains of four of them (the first, second, fourth, and fifth acidic residues, in italics), with the carbonyl oxygen groups of two non-conserved residues, are involved in calcium coordination. Thus mutations of these residues in the LDL receptor can result in folding defects of LDL-A modules and thus the overall structure of the LDL receptor, which indirectly lead to an impaired ligand-binding phenotype and eventually heart disease. However, the available biochemical and structural information on LDL-A modules and the ectodomain of the LDL receptor cannot explain why the third acidic residue is also highly conserved and why substitutions of this residue in different LDL-A modules of LDL receptor cause FH.

Tva is the cellular receptor for subgroup A of Rous sarcoma virus (RSV-A), and it is related to the LDL receptor superfamily because it contains a single LDL-A module of 40 amino acids in length within its extracellular region (13). Interaction between Tva and the RSV-A glycoprotein EnvA mediates viral entry. Tva specifically binds to the surface subunit (SU) of EnvA with high affinity, and this high affinity binding is important not only for viral attachment to the host cells but also important for receptor-triggered conformational changes on EnvA, which are essential for EnvA-mediated fusion between the viral and host membranes (14–17). Extensive molecular and biochemical analysis of Tva/EnvA interaction has demonstrated that the viral interaction domain of Tva is solely determined by the LDL-A module (18–22).

In this study, we examined the role of the third conserved acidic residue in protein folding and ligand binding using a model LDL-A module. This system is based on our previous work that demonstrated that the Tva LDL-A module can be functionally substituted by the human LDL receptor repeat 4 (hLDL-A4) with minor modifications (19). A unique and important aspect of this system is that several functional assays can be used to dissect the roles of individual residues in both protein folding and ligand recognition, in addition to the in vitro biochemical and structural analysis of individual LDL-A modules. This study reveals that the third conserved acidic residue, like other four acidic residues, is involved in calcium coordination and protein folding, providing...
a molecular explanation why this residue is conserved in the majority of hundreds of LDL-A modules, and why mutations of this residue in LDL receptor can lead to FH. Furthermore, binding kinetics analysis between the model LDL-A module and its ligand demonstrates that the second and third conserved residues of this LDL-A module are not directly involved in ligand binding. These results, although with potential caveats of a model system, should have broad implications in LDL-A module/ligand interactions.

EXPERIMENTAL PROCEDURES

DNA Methodology—A chimeric construct (referred to as Chimera in this study) was generated by replacing the C1–C3 region of TLA-G-A19L/D23H (19) with the corresponding region of the Tva LDL-A module by an overlapping PCR protocol. This construct was inserted into the LDL-A module of Tva using the myc-Tva backbone (19). The conserved acidic residues of Chimera were substituted with alanines either individually or in combinations (see Fig. 2A). All the constructs were confirmed by DNA sequencing. These constructs were used for examining protein expression and viral receptor function in human embryonic kidney 293T cells by transient transfection as described previously (18).

Protein Expression and Viral Infection Assay—Human embryonic kidney 293T cells were transiently transfected with DNAs of Chimera and its mutants by the CaPO4 method as described previously (18). The transfected cells were lysed with Triton X-100 48 h post-transfection, and protein expression of these constructs was examined by Western blotting following SDS-PAGE, using monoclonal antibody 9E10, which specifically recognizes the Myc tag portion of these proteins. Furthermore, to examine whether these proteins were expressed on the cell surface, transiently transfected 293T cells were subjected to flow cytometry. Briefly, 24 h post-transfection, cells were lifted, and 1 × 10⁶ cells were washed three times with FACS buffer (1× phosphate-buffered saline, 1% fetal bovine serum, and 0.1% sodium azide). Cells were resuspended in 100 μL of FACS buffer containing 1 μl of mouse anti-Myc-antibody 9E10 and incubated at 4 °C for 1 h. The cells were then washed and resuspended in 100 μl of FACS buffer containing 1 μl of goat anti-mouse antibody conjugated with fluorescein isothiocyanate and incubated at 4 °C for 1 h. Finally, cells were washed and resuspended in 500 μl of FACS buffer containing 1 μl of propidium iodide. The samples were collected and analyzed using a BD Biosciences FACSCalibur flow cytometer and Cellquest software.

To determine the viral receptor function of Chimera and its mutants, transiently transfected 293T cells were challenged with RCAS/A/AP, the recombinant RSV-A viruses, following a previous protocol (18). The alkaline phosphatase-positive 293T cells were detected under a microscope after staining, enumerated, and presented as number of alkaline phosphatase-positive cells per ml virus suspension used.

Expression and Characterization of the in Vitro Folding Properties of the LDL-A Proteins—The coding regions of the LDL-A modules of Chimera and its mutants were PCR-amplified using the DNA templates of these constructs in pcDNA-3. The amplified DNA fragments were digested with restriction endonucleases and cloned into pGEX-4T-1 (Amersham Biosciences), and the identity of each construct was confirmed by DNA sequencing. The LDL-A modules of Chimera and its mutants were expressed in Escherichia coli strain BL21 as glutathione S-transferase fusion proteins and purified by the GSH affinity column. After thrombin cleavage, the glutathione S-transferase portion was removed by the GSH affinity column, and the protein samples were further purified by reverse phase high performance liquid chromatography (HPLC) following a previous protocol established by us (23).

The purified proteins were refolded in the absence or presence of calcium following a published protocol (23), and the folding properties of the LDL-A proteins were examined by reverse phase HPLC and two-dimensional NMR spectroscopy. Its Ca²⁺ binding affinity was measured by isothermal titration calorimeter (Micocal MSC), following a previous protocol with minor modifications. The purified LDL-A proteins were refolded in the absence or in the presence of different concentrations of CaCl₂ (23). Following the refolding reactions, the samples were analyzed by reverse phase HPLC on a Vydac C4 column operated at a flow rate of 3.00 ml/min, using a linear gradient of 0.1% trifluoroacetic acid and 99% acetonitrile.

The ¹⁵N-labeled LDL-A proteins were used to acquire the [¹H-¹⁵N] HSQC spectra. Following the refolding step described above, each protein sample was eluted by reverse phase HPLC, and individual peaks were collected and prepared for acquisition of two-dimensional MNR spectra as described previously (23). Briefly, the NMR data were collected on a Bruker DRX600 spectrometer equipped with a pulse-field gradient accessory and operating at 600.13 MHz for ¹H and were processed and analyzed using Triad 6.3. The central frequencies were 4.70 and 118 ppm for ¹H and ¹⁵N, respectively.

Determination of the Binding Kinetics by IAsys—A resonant mirror biosensor (IAsys Auto+, Affinity Sensors) with a carbonyl cuvette was used to determine the kinetic constants (kₐ and k₈) and binding affinities (K_D) of the LDL-A proteins with the SUA-rIgG, which was transiently expressed in 293T cells and purified from the supernatant (17, 21). All the experiments were performed following a published protocol with modifications (17). Briefly, the LDL-A proteins were first refolded with calcium and then purified by reverse phase HPLC. The anti-SUA IgG well folded LDL-A proteins, determined by two-dimensional NMR, were prepared and immobilized to the [4-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl/N hydroxysuccinimide]-activated carboxylic cuvettes following the manufacturer’s protocol. The un-reacted N-hydroxysuccinimide esters and uncovered surface area were blocked by 2 mg/ml β-casein. The tightly associated but non-covalently bound protein was removed with 10 mM HCl. Binding of the SUA-rIgG protein to the immobilized LDL-A proteins was performed in phosphate-buffere d saline with additional 100 mM NaCl (to minimize the nonspecific binding) with 250 μM CaCl₂. The binding kinetics were examined by monitoring the association phase for 5 min and followed by monitoring the dissociation phase for 4 min. After each cycle, the cuvettes were regenerated with 100 mM HCl for 2–5 min. The binding cycle was repeated by using 6–8 different concentrations of the SUA-rIgG protein dissolved in phosphate-buffered saline, 100 mM NaCl with 250 μM CaCl₂. The experimental data were processed using FASTfit software (Affinity Sensor). The association constant (kₐ) was calculated from the gradient of the plot of apparent rate constant (kₐ) versus the SUA-rIgG protein concentration, which was obtained by a linear fit. The dissociation constant (k₈) was determined from the intercept of the k₈ versus [ligate] plot. When data were poorly fitted to a single exponential phase, a double exponential phase was used to process the association and dissociation data. K_D was obtained from K_D = k₈/kₐ.

Molecular Modeling of the Tva LDL-A Module—The newly proposed calcium-dependent conformation of the Tva LDL-A module, shown in Fig. 5D, was calculated using DYANA (24), a torsion angle dynamics annealing simulation program, following the same procedures as performed previously for the published structure of the Tva LDL-A module (25), shown in Fig. 5A.

RESULTS

Five Acidic Residues Are Highly Conserved at the C Terminus of the LDL-A Modules

Sequence analysis of the LDL-A modules from different members of the LDL receptor superfamily indicates that five acidic residues near the C terminus, DXXDXDXDXDE, are highly conserved among the LDL-A modules, and they will be referred to as D1, D2, D3, D4, and E5, respectively, in this report. Examination of 132 LDL-A modules from human LDL receptor, very low density lipoprotein receptor, LRP, complement, perlecain, sortilin-related receptor, corin, entokerinase, membrane serine proteases 1 and 2, and Tva, the RSV-A receptor, revealed that D4 and E5 are present in all of the modules and thus are invariant. In contrast, residue variations are found at the three remaining positions. Substitution of the aspartic acid by an asparagine accounts for all but one of the variations (25) at the D2 position. Although the total number of residue variations at the D2 and D3 positions is less than that at D1 (16 of 132 for D2, 9 of 132 for D3, respectively), other residues (His, Ser, Gln, Glu, Tyr, Thr, and Gly) in addition to asparagine, can be found at these positions. Fig. 1 shows the sequence alignment of 11 LDL-A modules. Because these positions are highly conserved among many LDL-A modules, it is reasonable to hypothesize that all of these acidic residues play important roles in LDL-A folding and/or ligand binding.

An important role for four of the five conserved acidic residues has been elucidated elegantly by the X-ray crystal structure of human LDL-A5 (10). Residues Asp-25 (D1), Asp-29 (D2),
Asp-35 (D4), and Glu-36 (E5) of LDL-A5 were shown to be involved in calcium coordination via their side chains, thus providing an explanation of why these acidic residues are conserved in LDL-A modules. In contrast, the role of D3 in LDL-A folding and function is not clear. Conservation of this residue in most LDL-A modules strongly suggests an important role. Furthermore, D3 mutations in human LDL receptor cause FH (6). These data support the notion that D3 plays an important role in protein folding and/or function of LDL-A modules.

Substitutions of Both the Second and Third Conserved Acidic Residues in the LDL-A Module Display a Defective Phenotype in Mediating RSV-A Entry

It has been demonstrated previously that the LDL-A module of Tva could be functionally replaced by the fourth LDL-A module (hLDL-A4) of human LDL receptor with a few substitutions to mediate RSV-A entry (19). In addition, the N-terminal region of the Tva LDL-A module (between cysteines 1 and 3) is also required for optimal receptor activity (26). Thus, a chimeric construct, referred to as Chimera in this study, was generated by appending the C1–C3 region of the Tva LDL-A module with the C3–C6 region of the modified hLDL-A4 module (Fig. 2A), using myc-Tva backbone plasmid. This chimeric protein is indistinguishable from the wt Tva in mediating efficient RSV-A viral entry (see Fig. 2B) and, importantly, retains all of the aforementioned conserved acidic residues.

Each of the five conserved acidic residues of Chimera, Asp-26 (D1), Asp-30 (D2), Asp-33 (D3), Asp-36 (D4), Glu-37 (E5), and Glu-32, which is not conserved among the LDL-A modules, was individually substituted by alanine (Fig. 2A). The effect of these mutations on RSV-A entry was determined by challenging the 293T cells transiently expressing these mutant proteins with the RCAS(A)-AP viral vector, a recombinant RSV-A virus that contains an alkaline phosphatase reporter gene (27). These results appear to show that these substitutions either allow the Chimera to mediate efficient viral entry or slightly lower the receptor function (see Fig. 2B).

Substitutions of Conserved Acidic Residues Result in Protein Folding Defects, HPLC Profiles

To characterize further the role of the conserved acidic residues in LDL-A folding, the LDL-A modules of Chimera and its mutants were expressed as glutathione S-transferase fusion proteins in Escherichia coli and purified by affinity chromatography as described under “Experimental Procedures.” Following thrombin cleavage and further purification, in vitro folding properties of the LDL-A modules of Chimera and its mutants were first examined by reverse phase HPLC after refolding in the presence or absence of calcium, following a protocol published previously.

The Chimera protein was eluted as multiple peaks when CaCl\(_2\) was omitted in the refolding. In contrast, the same protein was eluted as a single sharp peak (labeled as peak 2) as the CaCl\(_2\) concentrations were increased from 2 to 10 or 50 mM in the refolding step (Fig. 3). These results are consistent with the notion that the correct folding of Chimera, like that of Tva and the other LDL-A modules characterized previously by others and us, is calcium-dependent. Similarly, substitution mutant of a non-conserved acidic residue (E32A) of Chimera gave an almost identical HPLC profile as Chimera, suggesting that Glu-32 is unlikely involved in calcium coordination.

In contrast, the HPLC profiles of the substitution mutants of the five conserved acidic residues in Chimera showed that none of the substitutions affected the ability of Chimera to mediate efficient viral entry.

Three double substitution mutants (D30A/E32A, D30A/E33A, and E32A/D33A) were generated to further investigate the potential role of the second and third conserved acidic residues in RSV-A entry (Fig. 2A). When 293T cells transiently expressing these mutant proteins were challenged with the recombinant RSV-A viruses, only mutant D30A/D33A displayed slightly impaired viral receptor function (–3–4 logs lower than wt Tva). In contrast, the remaining two mutants (D30A/E32A and E32A/D33A) could still mediate efficient RSV-A entry at high DNA concentrations, and only displayed slightly lower receptor function at the lowest DNA concentrations (1 μg) in transfection (Fig. 2B). These results appear to suggest that the role of the second and third conserved acidic residues is redundant in mediating RSV-A entry. However, as demonstrated below, substitution of each of these residues adversely affects calcium-dependent protein folding. This discrepancy between the viral receptor function and protein folding of this Chimera will be explained under “Discussion.”

Total protein expression of Chimera and its mutants in 293T cells was examined by Western blotting, and it was found that all of the constructs were well expressed (data not shown). Furthermore, FACS analysis of surface expression of these proteins showed that they were all surface-expressed (data not shown), indicating that the defect of some Chimera mutants in mediating RSV-A entry is not due to problems of surface expression.
of these mutant proteins was eluted as a single sharp peak even as the CaCl₂ concentration was increased to 50 mM in the refolding step; instead, multiple peaks were eluted (Fig. 3). These results suggest that any individual substitution of these residues can adversely affect calcium-dependent protein folding. Because Asp-26, Asp-30, Asp-36, and Glu-37 have been implicated in calcium coordination, it is reasonable to assume that substitutions of these residues by alanines disrupted the native calcium cage of Chimera and thus resulted in protein misfolding. However, the elution profiles of mutants D30A and D33A are different from that of mutants D26A, D36A, and Glu-37. The proportion of peak fraction 4 (D30A) increased as more calcium was added in the refolding step (from 2 to 50 mM), whereas the percentage of other peaks such as peak fraction 5 became less prominent with higher concentrations of calcium. Similarly, the proportion of peak fraction 7 of D33A became more predominant than the other peaks (e.g. peak fraction 8) with higher concentrations of calcium. In contrast, peak fractions of 14 (D26A), 16 (D36A), and 18 (E37A) did not become so prominent even with higher calcium concentrations (Fig. 3). More important, as it will become clear in the two-dimensional NMR spectra (see below), the folding defect of D30A and D33A could be partially compensated by higher concentrations of calcium in the refolding step, and more than half of the protein samples (peak fractions 4 and 7, respectively) could be well folded in the presence of 50 mM CaCl₂. However, we could not detect any well folded fractions of D26A, D36A, E37A, or D30A/D33A mutant proteins under the same conditions.

Substitutions of Conserved Acidic Residues Result in Protein Folding Defects, Two-dimensional NMR Spectra

Two-dimensional [²H,¹⁵N]-HSQC NMR spectroscopy was used previously to demonstrate that Ca²⁺ is not only required for correct folding but also for maintaining the structural integrity of the Tva LDL-A module (23). Here we used the same technique to carefully examine the NMR spectra of 18 individual peak fractions indicated in Fig. 3 (numbered 1–18). The
NMR spectra, as shown in Fig. 4, can be roughly classified into three groups: 1) Chimera and E32A, 2) D30A and D33A, and 3) the remaining four mutants, D26A, D36A, E37A, and D30A/D33A. The major features of these classes are described below.

**Chimera and E32A**—When the proteins were folded in the absence of calcium, the peak fractions gave clustered [1H-15N]-HSQC NMR spectra (Fig. 4, A1 and E11), indicating that these peaks were not well dispersed. In contrast, when the proteins were folded in the presence of calcium, the peak fractions gave well dispersed spectra (A2 and E12), suggesting that these peaks were well structured. These results demonstrate that calcium is required for proper folding for Chimera and E32A proteins, as expected.

**D30A and D33A**—Like Chimera and E32A, when the proteins were folded in the absence of calcium, the peak fractions gave clustered NMR spectra (Fig. 4, B3 and C6). When the proteins were folded in the presence of calcium, peak fractions 4 (D30A) and 7 (D33A) gave well dispersed spectra (B4 and C7),

**Calcium-dependent LDL-A Folding**

![Fig. 3. Elution profiles of the Chimera and its mutant proteins by reverse phase HPLC. The purified proteins were refolded in the absence or presence of ramping concentrations of CaCl₂ (2, 10, and 50 mM, respectively) and subjected to reverse phase HPLC as described under “Experimental Procedures.” The individual peaks labeled in this figure were used for the two-dimensional NMR analysis shown in Fig. 4.](image-url)
FIG. 4. Two-dimensional [1H-15N] HSQC spectra of the Chimera and its mutant proteins. Two-dimensional [1H-15N] HSQC spectra of the purified, 15N-labeled protein peaks, labeled as 1–18 in Fig. 3 following elution by reverse phase HPLC, were acquired in the presence of various concentrations of CaCl$_2$ as described under “Experimental Procedures.” A, Chimera; B, D30A; C, D33A; D, D30AD33A; E, E32A; F, D26A; G, D36A; H, E37A.
These results suggest that substitutions of these acidic residues are specifically involved in ligand binding with very similar kinetics as Chimera and E32A. D30A and D33A gave $K_D$ values of 22.7 and 27.9 mM, respectively. These results indicate that Asp-30 and Asp-33 of Chimera are not directly involved in SUA binding. However, we were unable to determine whether the other three conserved acidic residues, Asp-26, Asp-36, and Glu-37, are directly involved in SUA binding because it was not possible to purify well folded mutant proteins of mutants D26A, D36A, or E37A (see Figs. 3 and 4).

**DISCUSSION**

In this study, we revealed the role of the third conserved acidic residue of a model LDL-A module in calcium-dependent protein folding and ligand recognition. This finding should have very broad implications in protein folding and function for all members of the LDL receptor superfamily. Furthermore, this finding provides a plausible explanation for a class of FH mutants that carry the substitutions of the third conserved acidic residue in the human LDL receptor. A unique aspect of this study is that we were able to use several functional assays to examine the role of each of the five conserved acidic residues of this module in mediating ligand recognition (EnvA binding) and viral infection (RSV-A entry). Because four of the five conserved acidic residues in the LDL-A module have been implicated in calcium coordination, we hypothesized that mutations of these residues would disrupt the proper folding and conformation of the LDL-A module, and the folding defect could be easily detected by the functional assays. Indeed, substitution of each of the three conserved acidic residues, Asp-26 (D1), Asp-36 (D4), or Glu-37 (E5), impaired the ability of the LDL-A module to efficiently mediate RSV-A entry (see Fig. 2). However, substitution of Asp-30 (D2) or Asp-33 (D3) did not appear to have any adverse effect on the LDL-A module in mediating RSV-A entry (see Fig. 2). Further analysis indicated that substitution of both Asp-30 and Asp-33 (D3) was required to abrogate the ability of the LDL-A module to mediate RSV-A entry, suggesting that Asp-30 and Asp-33 of the LDL-A module play a somewhat different role in protein folding from the other three conserved acidic residues.

The in vitro protein folding analysis revealed that both Asp-30 and Asp-33 are also involved in calcium-dependent protein folding, but they displayed unique properties in the HPLC elution profiles and the two-dimensional NMR spectra, generally consistent with the results of the viral entry assay. However, in stark contrast to the in vitro folding properties of

### Table I

| Immobilized Ligand | Ca $k_a$ | $k_d$ | $K_D$ |
|--------------------|---------|-------|-------|
|                   | $m^{-1} s^{-1} 10^4$ | $s^{-1} 10^{-9}$ | $m 10^{-9}$ |
| Chimera           | +       | 32.4 ± 2.4 | 6.7 ± 3.2 | 20.9 |
| E32A              | +       | 36.6 ± 1.4 | 5.8 ± 1.8 | 15.9 |
| D30A              | +       | 38.6 ± 3.5 | 8.8 ± 4.6 | 22.7 |
| D33A              | +       | 34.0 ± 2.3 | 9.5 ± 3.2 | 27.9 |
| Tva LDL-A+Ca$^+$  | +       | 160.0 ± 10.4 | 19.6 ± 8.8 | 12.3 |
| Tva LDL-A (−Ca$^+$) | −     | 9.7 ± 2.0   | 13.7 ± 2.9 | 140.6 |

Data are from Yu et al. (17). Ca, calcium.

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**Calcium-dependent LDL-A Folding**

**Substitutions of Conserved Acidic Residues Result in Protein Folding Defects, Calcium Binding Affinities**

Isothermal titration calorimetry was used to directly measure calcium binding affinities of Chimera and its mutant proteins following a published protocol (23). Calcium titrations were performed on a Microcal isothermal titration calorimeter. Chimera and E32A gave $K_D$ values of 72.7 ± 7.69 and 46.2 ± 7.43 mM, respectively. However, under the same conditions, none of the other Chimera mutant proteins displayed any detectable calcium binding activity probably due to the sensitivity limit of this assay (data not shown). Nevertheless, these results clearly demonstrated that substitution of any of the five conserved acidic residues in Chimera adversely affect calcium binding, consistent with the notion that all five conserved acidic residues are involved in calcium coordination.

**The Second and Third Conserved Acidic Residues Are Not Involved in Ligand Binding**

One interesting question is whether the conserved acidic residues of LDL-A modules are involved in ligand binding in addition to their role in calcium coordination and thus protein folding. This is not an easy question to answer because any substitution of the conserved acidic residues, as demonstrated above, can result in calcium-dependent folding defects, and therefore it is experimentally difficult to determine whether these acidic residues are specifically involved in ligand binding without structural analysis of LDL-A-ligand complexes. Nevertheless, the results described above provide a unique opportunity for us to investigate whether some of the acidic residues of Chimera are directly involved in EnvA binding.

Analysis of Chimera mutant proteins by HPLC and two-dimensional NMR demonstrated that a fraction of D30A and D33A mutant proteins were well folded in the presence of calcium. However, D26A, D36A, and E37A mutant proteins were not well folded under the same refolding conditions. Thus, it was possible for us to purify the well folded D30A and D33A proteins by HPLC (fraction peaks 4 and 7 of Fig. 3) and use these samples to measure the binding affinities to SUA-rIgG, as done by us previously (17) for Tva mutant proteins using IAsys. The peak fractions of Chimera, E32A, D30A, and D33A (fraction peaks 2, 12, 4, and 7 of Fig. 3) were collected and prepared for SUA-binding kinetic analysis using IAsys. The immobilized receptor peak samples were incubated with the SUA-rIgG protein, and the binding kinetics were determined in the presence of calcium as described under “Experimental Procedures.” Chimera and E32A proteins gave $K_D$ values of 20.9 and 15.9 mM, respectively, with similar values of $k_a$ and $k_d$ (Table I). E32A mutant displayed same binding kinetics as Chimera, indicating that Glu-32 is not involved in calcium coordination or ligand binding. Importantly, the well folded fractions of D30A and D33A proteins bound to SUA-rIgG with very similar kinetics as Chimera and E32A. D30A and D33A gave $K_D$ values of 22.7 and 27.9 mM, respectively. These results indicate that Asp-30 and Asp-33 of Chimera are not directly involved in SUA binding. However, we were unable to determine whether the other three conserved acidic residues, Asp-26, Asp-36, and Glu-37, are directly involved in SUA binding because it was not possible to purify well folded mutant proteins of mutants D26A, D36A, or E37A (see Figs. 3 and 4).
the substitution mutants of the other three conserved acidic residues, a fraction of the D30A and D33A mutant proteins could be correctly refolded in the presence of calcium, and the percentage of the correctly folded portion appeared to increase with higher concentrations of calcium (see Figs. 3 and 4). These results indicated that the folding defect of D30A and D33A mutant proteins could be partially compensated with high concentrations of calcium in the refolding steps. The same observation has been observed by others who demonstrated that the folding defect of a point mutation in human LDL receptor repeat 5 (D203G), which corresponds to the D3 position of the conserved acidic residue, could be compensated by higher concentrations of calcium (28), supporting the data in the current study. Together, these results strongly suggest that both D2 and D3, like the other three conserved acidic residues, are involved in calcium-dependent protein folding.

It should be pointed out that some discrepancies have been observed regarding the protein folding and function of the LDL-A module. The most dramatic difference is that a single mutation of either Asp-30 or Asp-33 did not show a noticeable effect on the receptor function of the LDL-A module, whereas both mutant proteins displayed a folding defect in vitro. However, this discrepancy can easily be reconciled by the following explanation. Tva expresses at a very low level in avian cells, but RSV-A can efficiently infect these cells, indicating that high expression of the receptor is not a prerequisite for efficient viral infection. In contrast, in the current study, the mutant proteins were highly expressed in mammalian cells by transient transfection. Overexpression of certain mutant proteins (like D30A or D33A) may mask the defect phenotype of these mutations, because a portion of the mutant proteins could be correctly folded in vivo and supported efficient RSV-A entry (20). As revealed by in vitro folding analysis, a fraction of these mutant proteins could be indeed correctly folded with higher calcium concentrations, consistent with our previous assumption.

We believe that the current study has important implications in elucidating the mechanism of protein folding and function of most, if not all, LDL-A modules. Because residues D2 and D3 displayed highly similar properties by both in vivo and in vitro analyses in this study, we can conclude that the third conserved acidic residue (D3), like the second one (D2), is also involved in calcium-dependent protein folding. We propose that each LDL-A module can adopt two calcium-dependent conformations. In one conformation, the side chains of D1, D2, D4, and E5 are involved in calcium binding as demonstrated by the x-ray structures of several individual LDL-A modules, whereas in another conformation, the side chains of D1, D3 (instead of D2), D4, and E5 coordinate calcium binding. In both conformations, the carbonyl oxygen groups of the two additional residues are also involved in calcium coordination. Here the Tva LDL-A module is used to illustrate the proposed two calcium-depend-
Calcium-dependent LDL-A Folding

In this study, we have demonstrated that a portion of D30A and D33A mutant proteins could be correctly folded in the presence of calcium in vitro, and the correctly folded portions could be separated from the other fractions and purified by reverse phase HPLC. Using IAsys, an optical biosensor, we have demonstrated that the correctly folded D30A and D33A mutant proteins bound to SUA with similar dissociation constants as Chimera (see Table I). These results clearly showed that neither residue Asp-30 nor Asp-33 is directly involved in ligand binding because it was not possible to obtain the correctly folded mutant proteins of these residues under the same experimental conditions. Therefore, the experiments in this study, for the first time, allowed us to exclude a role of Asp-30 and Asp-33 of the Chimera module in direct involvement of ligand interaction. Although these results should be interpreted with caution because they were derived from a model LDL-A module, these results may have important implications in LDL receptor/ligand interactions in general.

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