Identification of Amino Acid Residues That Determine pH Dependence of Ligand Binding to the Asialoglycoprotein Receptor during Endocytosis

(Received for publication, August 18, 1999, and in revised form, September 19, 1999)

Stephanie Wragg and Kurt Drickamer‡

From the Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

The rat hepatic asialoglycoprotein receptor mediates clearance of galactose- and N-acetylgalactosamine-terminated glycoproteins by endocytosis, binding ligands through a C-type, Ca\(^{2+}\)-dependent carbohydrate-recognition domain (CRD) at extracellular pH and releasing them at lower pH in endosomes. At physiological Ca\(^{2+}\) concentrations, the midpoint for ligand release from the CRD of the major subunit of the receptor is pH 7.1. In contrast, the midpoint is pH 5.0 for a galactose-binding derivative of the homologous C-type CRD of serum mannose-binding protein, which would thus not efficiently release ligand at an endosomal pH of 5.4. Site-directed mutagenesis of the CRD from the major subunit of the asialoglycoprotein receptor has been used to identify residues that are essential for efficient release of ligand at endosomal pH. The effects of changes to residues His\(^{256}\), Asp\(^{266}\), and Arg\(^{270}\) singly and in combination indicate that these residues reduce the affinity of the CRD for Ca\(^{2+}\), so that ligands are released at physiological Ca\(^{2+}\) concentrations. The proximity of these three residues to the ligand-binding site at Ca\(^{2+}\) site 2 of the domain suggests that they form a pH-sensitive switch for Ca\(^{2+}\) and ligand binding. Introduction of histidine and aspartic acid residues into the mannose-binding protein CRD at positions equivalent to His\(^{256}\) and Asp\(^{266}\) raises the pH for half-maximal binding of ligand to 6.1. The results, as well as sequence comparisons with other C-type CRDs, confirm the importance of these residues in conferring appropriate pH dependence in this family of domains.

The asialoglycoprotein receptor, which mediates clearance of galactose or N-acetylgalactosamine-terminated glycoproteins from circulation, is a prototype for receptor-mediated endocytosis. Related but distinct forms of the receptor are found in mammalian liver and in peritoneal macrophages (1, 2). The rat hepatic receptor is composed of three subunits, of which the major subunit, rat hepatic lectin 1 (RHL-1),\(^{1}\) represents 70–80% of the total mass of the receptor. The second subunit, RHL-2/3, consists of two species that are differentially glycosylated forms of a second, homologous polypeptide (3). RHL-1 and RHL-2/3 bind carbohydrate ligands through COOH-terminal, Ca\(^{2+}\)-dependent carbohydrate-recognition domains (C-type CRDs).

Structural analysis of wild-type and mutant forms of the homologous C-type CRD from serum mannose-binding protein suggests that carbohydrate ligands bind to the CRDs of RHL-1 at a conserved Ca\(^{2+}\) designated site 2. The primary interaction with sugar is through hydroxyl groups that form coordination bonds with the Ca\(^{2+}\) and hydrogen bonds with protein side chains that also ligate the Ca\(^{2+}\) (4). The molecular basis for galactose binding to C-type CRDs has been particularly well investigated in a mutant CRD from serum mannose-binding protein which has been engineered to bind galactose. Three single amino acid replacements (E185Q, N187D, and H189W) and insertion of a glycine-rich loop result in a modified CRD designated QPDWG, which has affinity and selectivity similar to RHL-1 (5). The crystal structures of this modified CRD and of a further mutant that binds N-acetylgalactosamine with high affinity have been solved (6, 7). These structures provide a basis for modeling the CRD of RHL-1.

Following binding of ligand to the asialoglycoprotein receptor at the cell surface, the receptor-ligand complex is endocytosed via clathrin-coated pits and directed to endosomes, where the complex dissociates. The carbohydrate ligand is targeted for degradation in lysosomes while the receptor recycles to the cell surface with a vacant binding site (1). Because the RHL-1 subunit alone can perform receptor-mediated endocytosis (8), it provides a useful tool for analyzing the molecular basis for the endocytosis process. Endosomal pH is an important determinant of recycling of the asialoglycoprotein receptor as well as other endocytic receptors (9). The dissociation process that occurs in endosomes can be mimicked in vitro by ligand release at pH 5.4. In the case of the chicken hepatic lectin, an homologous endocytic receptor, pH modulates structural transitions between several distinct states of the CRD (10). At endosomal pH, the structural change causes an approximately 10-fold reduction in affinity for Ca\(^{2+}\) with concomitant loss of ligand binding activity. Although these studies provide insight into the effect of pH on ligand binding activity and receptor conformation, they do not address the identity of the amino acids that sense the environmental pH.

The results of site-directed mutagenesis reported here identify three residues, including a histidine conserved in other endocytic receptors, that determine the pH dependence of ligand binding to RHL-1. Loss of ligand binding activity at low pH is closely correlated with decreased affinity for Ca\(^{2+}\).

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England BioLabs. Gal\(_\text{N}_{\text{ac}}\)-BSA was obtained from E-Y Laboratories and was iodinated by the chloramine-T method (11). Na\(_{125}\)I was purchased from Amersham Pharmacia Biotech. Galactose-Sepharose was prepared by

\(^{1}\) This work was supported by Grants 041845 and 054508 from the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

‡ Wellcome Principal Research Fellow. To whom all correspondence should be addressed: Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX1 3QU, United Kingdom. Tel.: 44-1865-275727; Fax: 44-1865-275339; E-mail: kd@glycob.ox.ac.uk.
the divinyl sulfone method (12). Immuno-4 polystyrene wells were from Dynex Technologies. DNA sequencing was performed using the Sequenase II kit from Amersham Pharmacia Biotech.

**Mutagenesis**—Mutagenesis was performed by insertion of double-stranded synthetic oligonucleotides (Gensys and Applied Biosystems, United Kingdom) at appropriate restriction sites of a CTD for RHL-1 as described previously (13). For expression, mutant constructs of RHL-1 were transferred to the pTST vector (14) and transformed into Escherichia coli strain C41(DE3), a modified version of strain BL21(DE3) (15). Procedures for the mutagenesis of a CTD for rat mannoside-binding protein and construction of modified expression vectors have been described (16).

**Protein Production**—Mutant versions of QPDWG were produced and isolated as described previously (17). Mutant versions of RHL-1 were prepared as described in Ref. 13, except that RHL-1 CRD production was induced in the transformed C41(DE3) strain with a final concentration of 0.7 mM isopropyl-β-D-thiogalactoside when the cells had grown to OD_{600} of 0.6. For fluorescence measurements, the mutant versions of QPDWG were further dialyzed to ensure the absence of Ca^{2+}. The proteins were dialyzed at 4 °C against three changes of 500 ml of 50 mM Tris-Cl, pH 7.8, 100 mM NaCl made with distilled water from which metal ions had been removed by passage over a Chelex-100 column.

**Solid Phase Binding Assays**—Each assay was performed in duplicate at least twice. Ca^{2+}-free BSA and incubation buffer were prepared by filtration through Chelex ion-exchange syringe filters (Bio-Rad). For the pH dependence assay, Immulon-4 wells were coated with protein and incubated at 4 °C overnight. After the protein solution was removed, the wells were rinsed twice with cold loading buffer (1.25 mM NaCl, 25 mM Tris-Cl, pH 7.8, 25 mM CaCl2) and blocked with 5% BSA in loading buffer for 2 h at 4 °C. The wells were rinsed twice with cold 1.25 mM NaCl, incubated for 10 min at 4 °C in the same solution and then rinsed a third time. Aliquots (50 μl) of 125I-Gal3-BSA (1 μg/ml) in 2 × incubation buffer (10 mM NaCl, 2 mM CaCl2, 0.5% BSA) were mixed with equal volumes of 2 × pH buffer (50 mM sodium acetate in the pH 4.5–6.5 range, 50 mM MES in the pH 6–7.5 range, and 50 mM Tris-Cl in the pH 6–8 range, with all solutions containing 2.5 mM NaCl and 4% BSA) and transferred to the Immulon-4 wells. Incubation was for 2 h at 4 °C, which was sufficient to achieve equilibrium binding. The wells were emptied, rinsed three times with cold high-Ca^{2+}-loading buffer (1.25 mM NaCl, 25 mM Tris-Cl, pH 7.8, 250 mM CaCl2), dried, and counted as above in a Wallac Wizard 1470 γ-counter.

For the determination of Ca^{2+} dependence of neoglycoprotein ligand binding, Immulon-4 wells were coated with protein and incubated at 4 °C overnight. One set of wells was prepared as described above and rinsed twice with cold high pH Ca^{2+}-free rinse buffer (1.25 mM NaCl, 25 mM Tris-Cl, pH 7.8) and a duplicate set of wells was rinsed with low pH Ca^{2+}-free rinse buffer (1.25 mM NaCl, 25 mM sodium acetate, pH 5.4). All wells were incubated for 10 min at 4 °C and then rinsed a third time in the appropriate Ca^{2+}-free rinse buffer. Aliquots (50 μl) of 125I-Gal3-BSA (1 μg/ml) in high pH, Ca^{2+}-free incubation buffer (1.25 mM NaCl, 25 mM Tris-Cl, pH 7.8, 5% BSA) or in low pH, Ca^{2+}-free incubation buffer (1.25 mM NaCl, 25 mM sodium acetate, pH 5.4, 5% BSA) were added to equal volumes of serial 3:2 dilutions of CaCl2 and then transferred to the appropriate Immulon-4 wells. Incubation was for 2 h at 4 °C. The wells were emptied, rinsed three times with cold high-Ca^{2+} loading buffer, dried, and counted as above.

**Fluorescence Measurements**—Measurements were performed on a Jasco FP777 fluorescence spectrophotometer with a 170 W lamp using a 1-cm square quartz cuvette. Emission spectra were recorded for 30 s using excitation at 295 nm (10 nm slit width) and measuring emission at 340 nm (5 nm slit width). For the determination of Ca^{2+} affinity, aliquots of CaCl2 were added to tubes containing 1.5 ml of a 1 μM protein solution in 50 mM sodium acetate, pH 5.5–6.2, 50 mM MES, pH 6.2–7.5, or 50 mM Tris-HCl, pH 6.7–7.8, with all solutions containing 100 mM NaCl. All buffers were made with distilled water passed over a Chelex-100 column. The tubes were allowed to equilibrate at 37 °C and the temperature of the measurements was maintained at 37 °C. Background intensity in samples without protein was subtracted from the measurements.

**Data Analysis**—Using a nonlinear least-squares fitting program (SigmaPlot, Jandel Scientific), data from the pH dependence assay were fitted to Equation 1.

\[
\text{Fraction maximal binding} = \frac{(pOH)_{\text{max}}}{pH_{\text{max}}} + (pOH)_{\text{max}} + (pOH)_{\text{max}}
\]

where \(pOH_{\text{max}}\) is the pH value at which half-maximal binding is observed. Data from the Ca^{2+} affinity binding assays and the fluorescence experiments were fitted to Equation 2,

\[
\text{Fraction maximal binding (or fluorescence)} = \frac{[Ca^{2+}]^2}{K_{Ca^{2+}}^2} + [Ca^{2+}]^2
\]

where \(K_{Ca^{2+}}\) is the concentration of Ca^{2+} at which half-maximal binding is observed. Values reported are the mean ± S.D. for at least two assays. Binding experiments were each performed in duplicate.

**Molecular Modeling**—The coordinates of QPDWG modified to contain histidine at residue 202 (7) were used for modeling RHL-1. Residues from the RHL-1 sequence were inserted at corresponding positions using the standard rotamer library in Insight II (BioSym). No attempt was made to minimize the structure.

**RESULTS**

**pH-dependent Binding of Ligands to C-type CRDs**—A solid-phase binding assay was used to analyze the pH dependence of ligand binding to the isolated CRD of RHL-1 (Fig. 1). At a Ca^{2+} concentration near to that found in extracellular fluids (1 mM), ligand binding to the isolated CRD decreases as the pH goes from extracellular, pH 7.3, to endosomal, pH 5.4. The pattern of binding is similar to that of the intact receptor (18), indicating that residues which confer pH sensitivity are located within the CRD portion of RHL-1. In contrast, QPDWG, the galactose-binding mutant CRD derived from serum mannoside-binding protein, shows substantially less change in ligand binding over the pH range 7.3 to 5.4, although ligand binding activity is lost at lower pH values. Similar behavior is observed for unmodified serum mannoside-binding protein, which is not directly involved in receptor-mediated endocytosis and therefore does not require the ability to release ligand at endosomal pH (data not shown). Points of half-maximal ligand binding, designated pH_{\text{pH}}, are 7.1 ± 0.1 for the CRD from RHL-1 and 5.0 ± 0.3 for QPDWG (averages for three different experiments).

The CRDs of QPDWG and RHL-1 are 31% identical in overall amino acid sequence and are based on the same protein fold (5). The difference in the behavior of these two CRDs suggests that pH sensitivity of ligand binding in the range between pH 5.4 and 7.3 is conferred by specific amino acid residues found in RHL-1 but not in QPDWG. Side chains that might be involved in receptor-mediated endocytosis and therefore does not require the ability to release ligand at endosomal pH (data not shown). Points of half-maximal ligand binding, designated pH_{\text{pH}}, are 7.1 ± 0.1 for the CRD from RHL-1 and 5.0 ± 0.3 for QPDWG (averages for three different experiments).

The CRDs of QPDWG and RHL-1 are 31% identical in overall amino acid sequence and are based on the same protein fold (5). The difference in the behavior of these two CRDs suggests that pH sensitivity of ligand binding in the range between pH 5.4 and 7.3 is conferred by specific amino acid residues found in RHL-1 but not in QPDWG. Side chains that might be involved in receptor-mediated endocytosis and therefore does not require the ability to release ligand at endosomal pH (data not shown). Points of half-maximal ligand binding, designated pH_{\text{pH}}, are 7.1 ± 0.1 for the CRD from RHL-1 and 5.0 ± 0.3 for QPDWG (averages for three different experiments).

The CRDs of QPDWG and RHL-1 are 31% identical in overall amino acid sequence and are based on the same protein fold (5). The difference in the behavior of these two CRDs suggests that pH sensitivity of ligand binding in the range between pH 5.4 and 7.3 is conferred by specific amino acid residues found in RHL-1 but not in QPDWG. Side chains that might be involved in receptor-mediated endocytosis and therefore does not require the ability to release ligand at endosomal pH (data not shown). Points of half-maximal ligand binding, designated pH_{\text{pH}}, are 7.1 ± 0.1 for the CRD from RHL-1 and 5.0 ± 0.3 for QPDWG (averages for three different experiments).
chicken hepatic lectin (10) and the macrophage asialoglycoprotein receptor (pH$_{H}$ of 6.3).  

On the assumption that pH sensitivity of ligand binding results from the interaction of protons with amino acid side chains, candidate residues for controlling pH sensitivity in RHL-1 were identified as residues conserved in the macrophage asialoglycoprotein receptor but not found in QPDWG. Comparison of the sequences shown in Fig. 2 reveals nine potentially protonatable residues that meet this criterion.

**Mutations in RHL-1 That Alter pH Sensitivity**—As an initial test of the role of specific residues in defining pH dependence of ligand binding to RHL-1, each of the nine residues identified in the preceding section was changed to a non-dissociating amino acid of a similar size. Changing Arg$^{273}$ to a leucine residue results in a CRD that can no longer be purified by affinity chromatography on galactose-Sepharose, suggesting that this mutation causes misfolding of the domain. The remaining eight mutants containing single site changes could be purified on galactose-Sepharose, indicating that the basic fold of the CRD is unaffected by these mutations. In the solid-phase binding assay, five of the mutants, with changes at positions His$^{203}$, Asp$^{224}$, Asp$^{227}$, Glu$^{229}$, and Asp$^{260}$, have profiles indistinguishable from wild-type (Table 1), suggesting that side chains of these residues are unlikely to play a role in establishing pH sensitivity of ligand binding.

Of the three single site mutations that do alter pH dependence of ligand binding, two cause a decrease in the pH$_{B}$ value. Representative results for mutants H256Q and D266N are shown in Fig. 3A. For both of these mutants, the pH that supports half-maximal ligand binding is approximately 1 unit lower than for wild-type CRD. The pH profile and pH$_{B}$ value for a double mutant containing both of these changes are very similar to those of the single mutants, indicating that His$^{256}$ and Asp$^{266}$ do not function in an additive fashion. The ability of the singly and doubly mutant CRDs to maintain ligand binding at endosomal pH also suggests that the role of His$^{256}$ and Asp$^{266}$ is to destabilize the CRD so that physiological release of ligand can take place.

A model for RHL-1 was generated based on the crystal structure of the CRD from a previously described variant of QPDWG that contains the histidine corresponding to His$^{256}$ in RHL-1. Insertion of the additional RHL-1 side chains that have been mutated reveals that the five residues which appear not to be involved in establishing pH dependence of ligand binding are at least 16 Å distant from the sugar-binding site adjacent to Ca$^{2+}$ site 2. In contrast, His$^{256}$ and Asp$^{266}$ are predicted to lie within 7 Å of this Ca$^{2+}$ (Fig. 4). The fact that the His$^{256}$ and Asp$^{266}$ side chains are close to each other is consistent with the idea that they work together to affect pH dependence and thus that mutations at these positions produce similar and non-additive effects. The proximity of these side chains to the sugar-binding site suggests that partial protonation of one of these residues might have a direct, local effect on the binding site, causing loss of ligand binding activity.

The remaining single mutant that displays a change in the pH dependence profile is R270L, for which the pH$_{B}$ is shifted to a higher value. This change is accompanied by a substantial loss in affinity for Ca$^{2+}$, so pH dependence was measured in the presence of 5 mM Ca$^{2+}$ (Fig. 3B). Mutation of the adjacent residue Arg$^{269}$ to leucine results in essentially no change in pH dependence of ligand binding, suggesting that the effect of the Arg$^{270}$ mutation results from a specific effect of this arginine side chain. Combination of the H256Q and D266N mutations with the R270L change results in partial compensation between the shifts seen with the separate mutations (Table 1). The loss in absolute affinity for Ca$^{2+}$ makes it difficult to interpret the effects of the change at position 270. However, as shown in Fig. 4, the arginine side chain is likely to be positioned close to His$^{256}$ and Asp$^{266}$ and within 8 Å of Ca$^{2+}$ site 2. It thus forms part of a cluster of amino acid side chains that affect pH sensitivity.

**Modulation of Ligand Binding by Ca$^{2+}$**—It has previously been demonstrated that pH-dependent binding of saccharide ligands to the chicken hepatic lectin is correlated with pH-dependent changes in affinity of the C-type CRD for Ca$^{2+}$ (10). The ability to generate mutant domains with altered pH dependence makes it possible to examine the relationship between pH and Ca$^{2+}$ binding in more detail. As a starting point, the Ca$^{2+}$ dependence of galactose-BSA binding to the wild-type CRD of RHL-1 and to QPDWG at high and low pH values are compared in Fig. 5. In all cases, binding is dependent on the second power of the concentration of Ca$^{2+}$, reflecting the presence of two Ca$^{2+}$ sites in the CRD (10). The Ca$^{2+}$ dependence can be characterized by an apparent K$_{Ca}$ which represent the Ca$^{2+}$ concentration that supports half-maximal ligand binding. Like the chicken hepatic lectin, RHL-1 shows substantially reduced affinity for Ca$^{2+}$ at endosomal pH. In contrast, QPDWG is relatively insensitive to a reduction in pH from 7.8 to 5.4.

The relationship between K$_{Ca}$ and pH over this range is demonstrated in greater detail for RHL-1 in Fig. 6. The value of log K$_{Ca}$ shows an approximately linear dependence on pH. The point at which this line crosses the physiological Ca$^{2+}$ concentration range represents the pH at which half-maximal binding of ligand will be achieved and thus corresponds to the pH$_{H}$ value. The behavior of the H256Q/D266N mutant of RHL-1 contrasts with the wild-type in that K$_{Ca}$ values at all pH values are lower. This shift has the effect of reducing the pH for half-maximal binding at physiological pH. For the receptor to function during endocytosis, the position of the line in Fig. 6 must be such that it passes through the physiological Ca$^{2+}$ concentration range between the pH value at the cell surface and the value in endosomes. As noted above, the effect of His$^{256}$ and Asp$^{266}$ is to decrease affinity for Ca$^{2+}$ over the entire pH range.

While it was not practical to measure K$_{Ca}$ values at all pH values for all of the mutant CRDs, the approximately linear relationship between pH and log K$_{Ca}$ makes it possible to gain an appreciation for the effects of the mutations by measuring K$_{Ca}$ at the two extreme pH values of 5.4 and 7.8. A compilation of such values for various mutant CRDs from RHL-1 is pre-

---

**Fig. 2.** Aligned amino acid sequences from CRDs of RHL-1 and other C-type lectins. Regions of secondary structure in QPDWG are indicated above the sequence. L denotes loop, S denotes β-strand, and H denotes α-helix. Residues coordinating the two Ca$^{2+}$ are denoted 1 and 2 above the sequence. The numbering corresponds to the RHL-1 sequence. Amino acids with potentially titratable side chains that are above the sequence. The numbering corresponds to the RHL-1 sequence.

---

2 H. Dobbyn and S. Wragg, unpublished observations.
Table I

| CRD                  | His<sup>256</sup> | Asp<sup>260</sup> | Asp<sup>266</sup> | Arg<sup>270</sup> | pH<sub>B</sub>(1 mM Ca<sup>2+</sup>) | pH<sub>B</sub>(5 mM Ca<sup>2+</sup>) | K<sub>Ca</sub>(pH 5.4) | K<sub>Ca</sub>(pH 7.8) |
|----------------------|-------------------|------------------|------------------|------------------|---------------------------|---------------------------|-----------------|-----------------|
| Mutations without effect on pH<sub>B</sub> |                   |                  |                  |                  |                           |                           |                 |                 |
| Wild-type RHL-1      | +                 | +                | +                | +                | 7.1 ± 0.1                 | 6.3 ± 0.1                 | 50.7 ± 0.2      | 0.49 ± 0.02     |
| H203Q                | +                 | +                | +                | —                | 7.1 ± 0.1                 | —                         | 65 ± 0           | 0.42 ± 0.00     |
| D224N                | +                 | +                | +                | —                | 6.8 ± 0.2                 | —                         | 92 ± 10          | 0.78 ± 0.11     |
| D227N                | +                 | +                | +                | —                | 6.9 ± 0.2                 | —                         | 96 ± 10          | 0.72 ± 0.07     |
| E229Q                | +                 | +                | +                | —                | 7.3 ± 0.1                 | —                         | 82 ± 1           | 1.10 ± 0.06     |
| D250N                | +                 | —                | +                | —                | 7.0 ± 0.1                 | —                         | 67 ± 12          | 1.18 ± 0.35     |
| R269L                | +                 | +                | +                | —                | 6.8 ± 0.2                 | —                         | 124 ± 7          | 0.85 ± 0.08     |
| Mutations that decrease pH<sub>B</sub> | —                 | +                | +                | +                | 5.9 ± 0.1                 | —                         | 15.4 ± 2.2       | 0.18 ± 0.02     |
| H256Q                | +                 | +                | +                | —                | 5.9 ± 0.3                 | —                         | 8.3 ± 0.9        | 0.07 ± 0.01     |
| H256Q/D266N          | —                 | +                | —                | +                | 6.0 ± 0.2                 | 5.5 ± 0.0                 | 4.2 ± 0.6        | 0.11 ± 0.01     |
| H256Q/D260N          | —                 | —                | +                | +                | 6.0 ± 0.1                 | —                         | 24.7 ± 3.2       | 0.29 ± 0.05     |
| D260/D266N           | +                 | —                | +                | —                | 5.8 ± 0.1                 | —                         | 12.1 ± 4.0       | 0.09 ± 0.03     |
| H256Q/D260N/D266N    | —                 | —                | —                | +                | 5.8 ± 0.1                 | —                         | 8.5 ± 1.2        | 0.13 ± 0.04     |
| Mutations that increase pH<sub>B</sub> | +                 | +                | +                | —                | NB<sup>5</sup>            | 8.6 ± 0.1                 | >500            | 11.3 ± 3.2      |
| R270L                | +                 | +                | +                | —                | 6.4 ± 0.1                 | —                         | 13.5 ± 0.1       | 0.38 ± 0.03     |
| H256Q/D266N/R270L    | —                 | +                | +                | —                | —                         | —                         | —               | —               |

<sup>a</sup> Not determined.

<sup>b</sup> NB, no binding at 1 mM Ca<sup>2+</sup>.

Fig. 3. pH dependence of ligand binding to wild-type and mutant forms of the CRD of RHL-1. Open and closed symbols represent data obtained in different buffer systems as described in the legend to Fig. 1. A, wild-type (circles), H256Q (squares), D260N (up triangles), H256Q/D260N (down triangles). B, wild-type (circles), R270L (octagons), H256Q/D260N/R270L (diamonds).

Fig. 4. Molecular model of RHL-1 showing the predicted positions of residues tested for involvement in determining pH dependence of ligand binding. The model was constructed based on the x-ray crystal structure of QPDWG mutant T202H complexed with N-acetylgalactosamine (Protein Data Bank code 1BCH). Carbon atoms are shaded black, oxygen white, and nitrogen gray. Large black sphere represents Ca<sup>2+</sup>. Four side chains, including Asn<sup>264</sup>, that form axial coordination bonds to Ca<sup>2+</sup> are shown. These four residues also form hydrogen bonds with the bound sugar ligand, which would be located above the plane of the page, directly above the Ca<sup>2+</sup>. Side chains of residues that alter the pH dependence of the CRD are also shown. The distance between Asn<sup>264</sup> and His<sup>256</sup>, indicated by the dashed line, is 2.97 Å. This figure was prepared with Molscript (19).
related to large changes in the relative $K_{Ca}$ values at pH 5.4 and 7.8, but represent an overall loss of affinity for Ca$^{2+}$ at all pH values.

This interpretation was verified in direct measurements of Ca$^{2+}$ binding, which can be monitored in mannose-binding protein and derivatives by following changes in intrinsic fluorescence (20). Results for QPDWG and the mutant with the highest measured pH$_B$ are shown in Fig. 7. As noted in previous experiments with wild-type mannose-binding proteins, the absolute $K_{Ca}$ values measured by this technique differ from those determined indirectly in the neoglycoprotein-binding assay, probably because of the effect of the bound sugar. Nevertheless, this approach provides a convenient way to compare affinity for Ca$^{2+}$ over a range of pH values. The results shown in Fig. 7 confirm that the mutations introduced into QPDWG result in an overall decrease in affinity for Ca$^{2+}$ at all pH values without substantially changing the relative affinity at low and high pH, which would correspond to increased slope in a plot of $K_{Ca}$ as a function of pH. Thus, even the mutant containing residues corresponding to His$^{256}$ and Asp$^{266}$, which has the highest pH$_B$ value at 1 mM Ca$^{2+}$, differs substantially from RHL-1 in its Ca$^{2+}$-binding properties.

In an attempt to make the behavior of QPDWG more like RHL, similar experiments were undertaken investigating the contribution of residues Arg$^{270}$ and Arg$^{270}$. The presence of a proline residue adjacent to Arg$^{270}$ in RHL-1 suggests that the local conformation of the polypeptide may differ substantially from QPDWG. For this reason, all three amino acid residues predicted to be in this loop (Arg-Pro-Tyr) were incorporated into QPDWG at positions 216–218. As indicated in Table II, this change by itself or in the presence of His$^{256}$ alters the pH only slightly when measured at 1 mM Ca$^{2+}$. However, the slight compensatory relationship between the His$^{256}$ and Arg$^{270}$ positions is amplified at 4 mM Ca$^{2+}$. These results are again consistent with the findings for RHL, in which removal of arginine at position 270 causes an increase in pH$_B$, since in QPDWG incorporation of the arginine residue decreases the pH$_B$. In contrast, in the presence of both Asp$^{266}$ and His$^{256}$, this arginine actually increases the pH$_B$. However, this triple mutant shows substantially reduced affinity for Ca$^{2+}$, again suggesting that changes in this region may result in significant rearrangement of the Ca$^{2+}$-binding sites. Similarly, mutants of QPDWG containing arginine at the position corresponding to Arg$^{273}$ of RHL-1 display greatly decreased affinity for Ca$^{2+}$, which could not be quantified using the solid-phase binding assay.

A further important difference between RHL and QPDWG is the arrangement of ligands for the accessory Ca$^{2+}$ site (site 1). As noted in Fig. 2, at least one of the site 1 ligands, at position 217 in RHL, must be different from QPDWG and two adjacent residues are absent from RHL. The effect of inserting the shorter loop corresponding to residues 216–219 of RHL, replac- ing residues 157–168 of QPDWG, was tested by the creation of a further important difference between RHL and QPDWG is the arrangement of ligands for the accessory Ca$^{2+}$ site (site 1). As noted in Fig. 2, at least one of the site 1 ligands, at position 217 in RHL, must be different from QPDWG and two adjacent residues are absent from RHL. The effect of inserting the shorter loop corresponding to residues 216–219 of RHL, replacing residues 157–168 of QPDWG, was tested by the creation of two additional mutations, resulting in a dramatic loss in affinity for Ca$^{2+}$ and a concomitant increase in the apparent pH$_B$ (Table II). These results suggest that the arrangement of Ca$^{2+}$ site 1 ligands may contribute to establishing the appropriate affinity for Ca$^{2+}$ and the pH$_B$ of RHL. However, the data also indicate that mutants containing the modified site 1 do not attain all of the Ca$^{2+}$-binding characteristics of RHL.

**FIG. 5.** Ca$^{2+}$ dependence of $^{125}$I-Gal-BSA binding to the wild-type CRD of RHL-1 and to QPDWG. Data were fitted to a second-order equation as shown by the solid lines. The buffers used were 25 mM Tris-HCl, pH 7.8 (closed symbols), and 25 mM sodium acetate, pH 5.4 (open symbols). A, wild-type RHL-1. B, QPDWG.

**FIG. 6.** pH dependence of apparent Ca$^{2+}$ affinity for wild-type and mutant CRD of RHL-1. $K_{Ca}$ values were derived from neoglycoprotein binding experiments as shown in Fig. 5 using three different sets of buffers as indicated in Fig. 1. Lines were fitted to the data based on the approximately linear relationship between log $K_{Ca}$ and pH. Shaded band represents the physiological Ca$^{2+}$ concentration range.
Ca²⁺ makes direct hydrogen bond interactions with certain of the QPDWG residues, using three different sets of buffers as indicated in Fig. 1. Residue in the QPDWG model background are known to vary their protonation state of the histidine side chain at position 256 determining the pH dependence of ligand binding to the CRD from RHL-1, indicating that loss of binding activity in the physiological pH range. Although the CRD of RHL-2/3, the minor subunit of the hepatic asialoglycoprotein receptor, is 59% identical to RHL-1, it binds Ca²⁺ more weakly and does not show a loss of affinity as the pH decreases from 7.8 to 5.4. Interestingly, this subunit differs at positions corresponding to His²⁵⁶, Asp²⁶⁶, and Arg²⁷⁰ (Fig. 2). These residues are also absent from almost all other CRDs in the C-type lectin family, which correlates with the fact that most of these proteins are not known to undergo pH-dependent release of ligand under physiological conditions.

CRDs that do contain a histidine side chain at the position corresponding to His²⁵⁶ of RHL-1 include the chicken hepatic lectin and the macrophage asialoglycoprotein receptor. The pH dependence of ligand binding to the chicken hepatic lectin is closely similar to RHL-1, although the amino acid residues corresponding to Asp²⁶⁶ and Arg²⁷⁰ are not conserved (Fig. 2), suggesting that, while the histidine side chain may remain the
depending on the presence of other side chains in this region (7), so it is not yet possible to propose a specific mechanism for linking the pH sensor to Ca²⁺ site 2. However, it is possible that a hydrogen bond forms between His²⁵⁶ and one of the site 2 Ca²⁺ ligands, corresponding to Asn²⁶⁴ (Fig. 4), so that protonation of the imidazole group would directly affect the position of the amide group and thus alter affinity for Ca²⁺. In any case, the divergent effects of changing residues Asp²⁶⁶ and Arg²⁷⁰ could result from the influence of these side chains on the pH sensitivities in the physiological pH range. Although the pH sensitivity of ligand binding for QPDWG and further mutant CRDs from serum mannose-binding protein

is probably mediated at least in part by this set of side chains acting as a pH sensor. The pH sensitivity of the domain is ultimately a result of the summed effect of multiple titratable groups in the domain. However, many potentially titratable groups on the surface of the protein can be mutated without affecting the pH dependence of ligand binding to the CRD from RHL-1, indicating that loss of binding activity in the physiological pH range is a result of a local event involving relatively few amino acid side chains.

One scenario consistent with the evidence is that the protonation state of the histidine side chain at position 256 determines the affinity for Ca²⁺ binding at site 2, perhaps because it makes direct hydrogen bond interactions with certain of the Ca²⁺ site 2 ligands. The exact interactions of this histidine residue in the QPDWG model background are known to vary depending on the presence of other side chains in this region (7), so it is not yet possible to propose a specific mechanism for linking the pH sensor to Ca²⁺ site 2. However, it is possible that a hydrogen bond forms between His²⁵⁶ and one of the site 2 Ca²⁺ ligands, corresponding to Asn²⁶⁴ (Fig. 4), so that protonation of the imidazole group would directly affect the position of the amide group and thus alter affinity for Ca²⁺. In any case, the divergent effects of changing residues Asp²⁶⁶ and Arg²⁷⁰ could result from the influence of these side chains on the pH

| Amino acid changes | His²⁵⁶ | Asp²⁶⁶ | Asp²⁶⁶ | Arg²⁷⁰ | pHₐ (1 mM Ca²⁺) | pHₐ (4 mM Ca²⁺) | Kᵥₑ (pH 5.4) | Kᵥₑ (pH 7.8) |
|--------------------|-------|-------|-------|-------|----------------|----------------|-------------|-------------|
| QPDWG +            | –     | –     | –     | –     | 5.0 ± 0.3      | 3.5 ± 0.1      | 0.98 ± 0.09 | 0.42 ± 0.04 |
| I212D              | –     | –     | +     | –     | 5.2 ± 0.1      | –              | 1.34 ± 0.06 | 0.31 ± 0.04 |
| N206D              | –     | –     | –     | –     | 5.1 ± 0.1      | –              | 1.46 ± 0.07 | 0.13 ± 0.02 |
| T202H              | –     | –     | –     | –     | 5.7 ± 0.1      | 5.3 ± 0.1      | 2.65 ± 0.3  | 0.17 ± 0.03 |
| T202H/N206D        | +     | –     | –     | –     | 5.7 ± 0.1      | –              | 3.96 ± 1.0  | 0.08 ± 0.02 |
| T202H/I212D        | +     | –     | –     | –     | 6.1 ± 0.1      | 5.7 ± 0.1      | 3.28 ± 0.5  | 1.4 ± 0.2   |
| T202H/N206D/I212D  | +     | +     | –     | –     | 5.9 ± 0.1      | –              | 5.37 ± 0.6  | 1.3 ± 0.5   |

QPDWG in which region 216–218 is replaced by RHL-1 sequence (RPY) +

| Amino acid changes | His²⁵⁶ | Asp²⁶⁶ | Asp²⁶⁶ | Arg²⁷⁰ | pHₐ (1 mM Ca²⁺) | pHₐ (4 mM Ca²⁺) | Kᵥₑ (pH 5.4) | Kᵥₑ (pH 7.8) |
|--------------------|-------|-------|-------|-------|----------------|----------------|-------------|-------------|
| I212D              | –     | –     | +     | –     | 5.3 ± 0.0      | 3.2 ± 0.0      | 0.67 ± 0.2  | 0.08 ± 0.02 |
| N206D              | –     | –     | –     | –     | 5.7 ± 0.1      | 4.8 ± 0.2      | 1.47 ± 0.1  | 0.55 ± 0.2  |
| T202H              | +     | –     | –     | –     | 5.5 ± 0.0      | 4.7 ± 0.4      | 2.27 ± 0.3  | 0.16 ± 0.03 |
| T202H/I212D        | +     | +     | –     | –     | NB             | 6.4 ± 0.1      | 28.2 ± 5.7  | 7.2 ± 0.3   |

QPDWG in which region 157–161 is replaced by RHL-1 sequence (QNG) +

| Amino acid changes | His²⁵⁶ | Asp²⁶⁶ | Asp²⁶⁶ | Arg²⁷⁰ | pHₐ (1 mM Ca²⁺) | pHₐ (4 mM Ca²⁺) | Kᵥₑ (pH 5.4) | Kᵥₑ (pH 7.8) |
|--------------------|-------|-------|-------|-------|----------------|----------------|-------------|-------------|
| Q162P              | –     | –     | –     | –     | 7.1 ± 0.1      | –              | 37.53 ± 6.2 | 4.75 ± 0.06 |
| Q162P              | –     | –     | –     | –     | 7.8 ± 0.2      | –              | 72.95 ± 1.4 | 3.69 ± 0.9  |

* Position of the equivalent residues in the CRD of RHL-1.
* Not determined.
* NB, no binding at 1 mM Ca²⁺.
primary pH sensor, the effective $pK_a$ of this side chain is established in different ways. The absence of the critical histidine residue from the primary ligand-binding CRD of the macrophage mannose receptor (CRD-4) correlates with the fact that ligand release from this CRD occurs at lower pH than from RHL-1 (21).

Other endocytic receptors share with the C-type lectins the presence of divalent cations at critical sites in the ligand-binding domains. The Mn$^{2+}$ residue that forms part of the ligand-binding site in the cation-dependent mannose 6-phosphate receptor is in close proximity to a histidine residue that has been suggested as a possible pH-dependent regulator of sugar binding (22). Many other endocytic receptors, such as the low density lipoprotein receptor, contain homologous complement-like repeats that are folded around a Ca$^{2+}$. Several of these domains have been characterized structurally and it has been suggested that loss of ligand binding activity at endosomal pH may be mediated by release of the Ca$^{2+}$ (23). However, examination of the sequences of the Ca$^{2+}$-containing complement-like repeats fails to reveal a conserved histidine residue near to the Ca$^{2+}$ site. In different receptors, other mechanisms must be responsible for determining the pH-sensitive changes in ligand binding activity that are essential to the endocytic processes. In addition, the concentration of Ca$^{2+}$ in endosomes appears to be reduced compared with the extracellular environment, providing an alternative route to destabilization of Ca$^{2+}$-dependent receptor-ligand complexes (24).

Acknowledgments—We thank Ken Ng and Bill Weis for help with the fluorescence experiments.

REFERENCES
1. Spiess, M. (1990) *Biochemistry* **29**, 10008–10019
2. Li, M., Kurata, H., Itoh, N., Yamashina, I., and Kawasaki, T. (1990) *J. Biol. Chem.* **265**, 11295–11298
3. Halberg, D. F., Wager, R. E., Farrell, D. C., Hildreth, J., IV, Quesenberry, M. S., Loeb, J. A., Holland, E. C., and Drickamer, K. (1987) *J. Biol. Chem.* **262**, 9828–9838
4. Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) *Nature* **360**, 127–134
5. Iobst, S. T., and Drickamer, K. (1994) *J. Biol. Chem.* **269**, 15512–15519
6. Kolatkar, A., and Weis, W. I. (1996) *J. Biol. Chem.* **271**, 6679–6685
7. Kolatkar, A. R., Leung, A. K., Isecke, R., Brossmer, R., Drickamer, K., and Weis, W. I. (1998) *J. Biol. Chem.* **273**, 19502–19508
8. Braiterman, L. T., Chance, S. C., Porter, W. R., Lee, Y. C., Townsend, E. R., and Hubbard, A. L. (1989) *J. Biol. Chem.* **264**, 1682–1688
9. Mellman, I., Fuchs, R., and Helenius, A. (1996) *Annu. Rev. Biochem.* **65**, 663–700
10. Loeb, J. A., and Drickamer, K. (1988) *J. Biol. Chem.* **263**, 9752–9760
11. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
12. Fornstedt, N., and Porath, J. (1975) *FEBS Lett.* **57**, 187–191
13. Iobst, S. T., and Drickamer, K. (1996) *J. Biol. Chem.* **271**, 6686–6693
14. Eisenberg, S. P., Evans, R. J., Arend, W. P., Verderber, E., Brewer, M. T., Hannum, C. H., and Thompson, R. C. (1990) *Nature* **343**, 341–346
15. Miroux, B., and Walker, J. E. (1996) *J. Mol. Biol.* **260**, 289–298
16. Iobst, S. T., Wormald, M. R., Weis, W. I., Dwek, R. A., and Drickamer, K. (1994) *J. Biol. Chem.* **269**, 15505–15511
17. Weis, W. I., Crichlow, G. V., Murthy, H. M. K., Hendrickson, W. A., and Drickamer, K. (1991) *J. Biol. Chem.* **266**, 20678–20686
18. Hudgin, R. L., Price, W. E., Jr., Ashwell, G., Stockert, R. J., and Morell, A. G. (1974) *J. Biol. Chem.* **249**, 5536–5543
19. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
20. Ng, K. K.-S., and Weis, W. I. (1998) *Biochemistry* **37**, 17977–17989
21. Mullin, N. P., Hall, K. T., and Taylor, M. E. (1994) *J. Biol. Chem.* **269**, 28405–28413
22. Roberts, D. L., Weix, D. J., Dahms, N. M., and Kim, J.-J. P. (1998) *Cell* **93**, 639–648
23. Domer, K., Huang, W., and Gettins, P. G. W. (1998) *Biochemistry* **37**, 17016–17023
24. Gerasimenko, J. V., Tepikin, A. V., Petersen, O. H., and Gerasimenko, O. V. (1998) *Curr. Biol.* **8**, 1335–1338