Comparative Analysis by Two-Dimensional Iodopeptide Mapping of the RhD Protein and LW Glycoprotein

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The RhD polypeptide and LW glycoprotein were separately immunopurified with monoclonal antibodies and compared by two-dimensional (2-D) iodopeptide mapping after digestion with α-chymotrypsin. These proteins have distinct 2-D maps, as seen after 125I-labeling tyrosine residues (chloramine-T procedure), and even more strikingly after labeling primary amine residues (Bolton-Hunter procedure). Of the more than 20 iodopeptides visualized, only five migrated identically when preparations of RhD and LW were directly compared, suggesting that RhD and LW are different proteins that may share some common protein domains. N-glycanase treatment of the iodopeptides did not modify the 2-D map of the RhD protein but greatly affected the LW map, further indicating that LW, but not RhD, carries N-linked carbohydrate chains. After deglycosylation the LW map was different from the RhD map, confirming that the RhD and LW polypeptides are different proteins. These findings demonstrate that LW is neither a glycosylated form of Rh protein nor is Rh a precursor of LW.

To further define the chemical relationship between Rh and LW, we have immunopurified these components from human erythrocytes and compared their structure by 2-D peptide mapping.

**EXPERIMENTAL PROCEDURES**

Packed erythrocytes from blood units were filtered through a Sepacell R-500 leukocyte removal filter (Fenwal Laboratories, Deerfield, IL), and membranes were prepared as described. The RhD polypeptide and the LW glycoprotein were purified by large scale immunoprecipitation from cDE/cDE erythrocyte membrane vesicles using monoclonal antibodies (MoAbs) against RhD and LW antigens, respectively, as described previously. About 60 μg of each polypeptide as precipitated with cold acetone and solubilized in 120 μL of 6% wt/vol sodium dodecyl sulfate (SDS) and 50 mmol/L borate, pH 8. About 30 μg of polypeptides were labeled with 125I-Na by the chloramine-T procedure as described. The remaining material was labeled with 500 μCi of Bolton-Hunter reagent (N-succinimidyl-3-[4-hydroxy-5(125I-iodophenyl)] propionate) according to manufacturer’s instructions (Amersham Corp, Arlington Heights, IL). Free iodine was separated from labeled proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to manufacturer’s instructions. There was no degradation of the polypeptides during the process, and the 32,000 molecular weight (mol wt) 125I-labeled RhD protein and the 44,000 mol wt LW glycoprotein were localized in the gel by autoradiography and excised. The 2-D iodopeptide mapping was performed basically as described. Gel slices containing the 125I-labeled RhD and LW polypeptides were washed extensively with 25% (vol/vol) isopropanol and then with 10% (vol/vol) methanol, dried in a vacuum speed centrifuge in siliconized glass tubes, and incubated overnight at 37°C in 2 mL of 50 mmol/L NH₄HCO₃.

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containing 200 μg of proteolytic enzyme (α-chymotrypsin, 43 U/mg, or trypsin-DCC treated, 33 U/mg). The supernates were dried under vacuum, and one part of the iodopeptides was directly dissolved in electrophoresis buffer (acetic acid, formic acid, H₂O; 15:5:80). The other part was digested for 18 hours with 10 U/mL N-glycanase (Boehringer, Mannheim, FRG) at 37°C in 20 mmol/L sodium phosphate pH 8.6, 10 mmol/L EDTA, 0.5% β-mercaptoethanol, and 1% (wt/vol) Triton X-100. The treated iodopeptides were precipitated twice with 80% trichloroacetic acid (TCA), washed in 70% methanol, dried under vacuum, and dissolved in the electrophoresis buffer as described above. The different samples were spotted on 20 × 20 cm cellulose plates (Merck, Darmstadt, FRG) and subjected to electrophoresis at 1,000 V for about 1 hour. The plates were air-dried before thin layer chromatography (butanol, pyridine, acetic acid, H₂O; 64:50:10:40). Iodopeptides were visualized by exposure to Kodak AR 5 X-Ray film (Eastman Kodak, Rochester, NY) for 2 to 24 hours. Iodopeptides of identical mobility on the Rh and LW maps were scraped from the thin-layer plates, eluted in 50 μL of electrophoresis buffer at 22°C for 4 hours, and reanalyzed in two dimensions as above, either separately or as a mixture.

RESULTS AND DISCUSSION

The 2-D chymotryptic iodopeptide maps of Rh and LW polypeptides obtained after labeling the tyrosine residues are shown in Fig 1. A total of 26 and 19 major iodopeptides were detected in the digests of the LW and RhD polypeptides, respectively (Fig 1, A and D). The 2-D map of the RhD

![Image A](image-a)

![Image B](image-b)

![Image C](image-c)

![Image D](image-d)

**Fig 1.** 2-D iodopeptide maps of RhD and LW polypeptides ¹³C-labeled on tyrosine residues. RhD and LW polypeptides were precipitated from sDE/sDE erythrocyte membranes using MoAbs specific for RhD and LW. Iodopeptide maps from LW glycoprotein and RhD protein are shown in panels A and D, respectively. Panel C shows a composite drawing with the LW and RhD iodopeptides indicated as light and heavy outlines, respectively. S and s1 to s4 refer to cell surface labeled peptides of Rh and LW, respectively (see text). Peptides 1, 3, 4, 5, and s1 to s3 are not modified in position after treatment of LW iodopeptides with N-glycanase, as shown in panel B.
polypeptide (Fig 1D) was nearly identical to that determined previously. The 2-D map of iodopeptides derived from the LW glycoprotein has not been previously seen and showed significantly more peptides, most migrating in a position different from the iodopeptides in the RhD map (Fig 1A). These differences were not related to efficiency of iodopeptide release from the gel, since 80% and 60% of the applied radioactivity was recovered in the supernates of RhD and LW chymotryptic digests, respectively.

The major RhD and LW iodopeptides labeled at the extracellular membrane surface were also identical with those determined previously and are indicated by S and s1 to s4 on the composite map of RhD and LW, respectively (Fig 1C).

The positions of the RhD iodopeptides were not altered by N-glycanase treatment (not shown), a finding in agreement with previous observations indicating that the Rh polypeptides are not significantly glycosylated. On the contrary, the position of at least 12 to 15 LW iodopeptides were modified by this treatment (Fig 1B), whereas seven others (notated 1, 3, 4, 5, and s1 to s3, Fig 1C) remained unchanged, therefore indicating that several iodopeptides of the LW map carry N-glycans. This is consistent with our previous estimation of 8 to 9 N-glycan chains attached per molecule of LW glycoprotein.

It is obvious also that the 2-D map of the deglycosylated LW glycoprotein is different from the 2-D map of the RhD polypeptide, a finding that clearly demonstrates that LW is neither a glycosylated form of Rh nor is Rh a precursor of LW. This agrees well with our present knowledge, indicating that Rh and LW are genetically independent systems encoded by genes located on different chromosomes (1 and 19, respectively). Our findings also indicate that the LW glycoprotein defect of Rhnull cells cannot be explained by a lack of Rh substrate for LW synthesis. Rather, it is thought that the Rh and LW proteins, as well as other membrane polypeptides that are lacking or

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**Fig 2.** Reanalysis of selected Rh and LW iodopeptides. Iodopeptides of similar mobility (numbered as regions 1, 2, s2, s3, and s4 on Fig 1C) on 2-D maps of Rh and LW were scraped from the thin-layer plates, eluted, and reanalyzed on 2-D maps either separately (Rh, LW) or as a mixture (Rh + LW). Only relevant areas of the second 2-D maps are shown. Arrows indicate peptides of identical mobility contained within region 1 defined in Fig 1C. Peptides 2, s2, and s3 comigrate in the Rh-LW mixture, whereas peptides s4 of Rh and LW are different.
severely depressed in Rhnull or Rhmod cells,24-27 are all noncovalently associated within the cell membrane as a structural aggregate termed the Rh cluster.28 It is postulated that the defect of a single component of this cluster (most likely a Rh protein) may destabilize the structure, thereby preventing insertion of the other membrane components (eg, LW) into the phospholipid bilayer. This model is substantiated by immunoprecipitation experiments showing that several proteins of the Rh cluster can be coprecipitated with some MoAbs.21,29

Comparative analysis of the 2-D maps also demonstrated that at least five iodopeptides (three peptides noted as 2, s2, s3, and two peptides in the region noted 1 on Fig 1C) of the 19 Rh and 26 LW iodopeptides migrated identically, suggesting that they may represent identical or very similar peptide species. This was further demonstrated by extraction of these peptides from the Rh and LW thin-layer plates and reanalysis in the 2-D systems, either separately (Rh, LW) or as a mixture (Rh + LW) as shown in Fig 2. We found that two peptides within region 1 comigrated in the Rh and LW mixture (arrows on Fig 2), as did the three peptides 2, s2, and s3. In contrast, peptides s4 from Rh and LW migrate at a slightly different position on reanalysis. Peptide s3 from LW is surface-exposed but is probably not significantly glycosylated, since it was still present after N-glycanase treatment of the LW iodopeptides (Fig 1B). On the contrary, peptide s4 from LW is also cell surface-exposed but is glycosylated, since it is absent after N-glycanase treatment (Fig 1B). Although the iodopeptide S from RhD comigrated with an LW iodopeptide (Fig 1C), these peptides are not identical, since the latter is N-glycosylated and disappeared after N-glycanase treatment.

The 2-D chymotryptic iodopeptide maps obtained after labeling the primary amine residues are shown in Fig 3. There is a striking difference between the maps of LW and RhD proteins analyzed this way, since only two major spots were present on the RhD map as compared with 16 to 20 on the LW map. These differences were also noted on RhD and LW maps performed after tryptic cleavage (not shown). These findings further demonstrate that the RhD and LW polypeptides are different molecules and indicate also that protease-sensitive primary amine residues of the RhD polypeptide22 may be clustered on this protein, possibly within two small domains that extend above or below the lipid bilayer. This would be consistent with the physical behavior of the Rh protein, which exists almost entirely between the leaflets of the bilayer.17 On the contrary, the primary amine residues of the LW glycoprotein are probably distributed throughout the molecule.

It is interesting that region 1 of the 2-D maps includes peptides carrying tyrosine and primary amine residues, as shown from radiolabeling (Figs 1C and 3), which is consistent with the hypothesis that this area contains two peptides very similar in Rh and LW proteins. However, peptide s2 is also common to both proteins (Fig 2) but contains only radiodinated tyrosine residues. The overall homology thus detected between RhD and LW polypeptides by 2-D mapping analysis of chymotryptic iodopeptides is limited to five peptides, out of approximately 20 major spots present in each map. This suggests that these proteins, although different, share peptide segments that may have a common origin. It is possible that the degree of homology between these proteins is even greater, since 2-D map analysis accentuates differences between proteins and since a single amino acid substitution may drastically modify the migration position. It is interesting and probably significant that region 1, which includes two peptides shared by RhD and LW proteins (Figs 1C and 2), has also been found to be conserved among the Rh-related polypeptides present in nonhuman erythrocytes.8 Whether these peptides are important for some structural or physiologic function of these proteins requires further studies.

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