Human erythrocyte p55 is a peripheral membrane protein that contains three distinct domains in its primary structure: an N-terminal domain, an SH3 motif, and a C-terminal guanylate kinase domain. We used naturally mutated red blood cells (RBCs) with primary genetic defects resulting in the absence of protein 4.1 (4.1(-) hereditary elliptocytosis) or glycophorin C (Leach elliptocytosis). The absence of either protein was associated with the absence of p55. On a stoichiometric basis, the reduction in glycophorin C (about 80%) was concomitant to the lack of p55 in RBCs devoid of protein 4.1. Similarly, the reduction of protein 4.1 (about 20%) was equivalent to the absence of p55 in RBCs devoid of glycophorin C. These correlations suggest that p55 is associated, in precise proportions, with the protein 4.1–glycophorin-C complex, linking the skeleton and the membrane. The protein 4.1–glycophorin-C cross-bridge is known to be critically important for the stability and mechanical properties of human RBC plasma membrane. Because isoforms of protein 4.1, glycophorin C, and p55 exist in many tissues, these results provide evidence of a linkage between the skeleton and the membrane that may have implications in many nonerythroid cells.

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Evidence That Red Blood Cell Protein p55 May Participate in the Skeleton-Membrane Linkage That Involves Protein 4.1 and Glycophorin C

RESULTS

In homozygous 4.1(-) HE individuals KB and SA II.9, presenting with a primary absence of protein 4.1, RBCs exhibited a 70% to 90% reduction of GPC.10-13 Denstometric scanning of Coomassie-stained gels of RBC membrane proteins also indicated the absence of a 55-Kd protein (Fig 1). The absence of a 55-Kd band had been previously noted in these erythrocytes.10-12 Upon Western blot analysis of RBC membrane proteins with anti-p55 antibodies, p55 turned out to be missing (Fig 2, lanes b and c).

In heterozygous 4.1(-) HE, the reduction of p55 was proportional to that of protein 4.1 (Table 1 and Fig 1). In this condition, the reduction in GPC content is similarly proportional to the reduction of protein 4.1, as previously reported.12,13

We found an approximately 20% decrease of protein 4.1 in Leach elliptocytes displaying a primary absence of GPC and GPD (Table 1 and Fig 1). This reduction of protein 4.1, not previously mentioned,13 was highly significant and approached the reduction of protein 4.1 observed in those 4.1(-)-HE heterozygotes that show a low-key decrease of protein 4.1 (individual DU II.1, Table 1). The similarity of phenotypes between heterozygous 4.1(-) HE and Leach HE, which display comparable amounts of protein 4.1 but different amounts of GPC, suggests that protein 4.1 may be functionally more important than GPC in the genesis of elliptocytosis.

Most strikingly, the Leach elliptocytes also showed the lack of p55 (Table 1 and Figs 1 and 2). However, a faint signal of intact p55 was detectable in overdeveloped Western blots of samples from individual LN but not from individual MWB (Fig 2). In fact, this observation correlated with the existence of trace amounts of a truncated form of GPC in the RBCs of individual LN. The truncated GPC contains only the transmembrane and cytoplasmic domains and is missing a portion of the extracellular domain. This may arise from a ribosomal reinitiation on the mutated cyto p55.4

In Gerbich deficient individual O11.DO [Ge(-) type], who presented with no elliptocytosis, the amount of protein 4.1 was normal12 (Fig 1). The p55/4.1 ratio (15.5 ± 12 ± 2 [n = 5] in controls) also indicated a normal content in p55 in these erythrocytes.

DISCUSSION

Stoichiometric implication. There are about 200,000 copies of protein 4.1 per RBC.32 Given the hexagonal and pentagonal arrangement of spectrin in the skeleton,33,34 there are approximately 30,000 junctional complexes per erythrocyte. Hence, there are approximately 6 molecules of protein 4.1 per complex. The amount of GPC is about two- to fourfold lower than that of protein 4.1.16,19 Therefore, it appears that only a subset of protein-4.1 molecules may participate in the attachment of the skeleton to the membrane via GPC. As a matter of fact, our data suggest that only 20% (approximately 40,000 molecules) of protein 4.1 bind to GPC, considering the fraction of protein 4.1 (20%) that lacks in Leach HE. In addition, it is known that protein 4.1 binds to other transmembrane proteins, such as band 3,35-37 glycophorins A and B,38 and spectrin and actin.39,40

The fact that p55 is absent in human erythrocytes when either protein 4.1 or GPC are missing suggests that the amount of p55 on the membrane is limited by both protein 4.1 and GPC. Reciprocally, we propose that p55 plays a role in the protein-4.1-GPC interaction on the plasma membrane. The following stoichiometric considerations are consistent with this view. In Leach HE, the primary absence of GPC causes a 20% loss of protein 4.1 (approximately 40,000 molecules) and a loss of all p55 molecules (approximately 40,000 dimers).4 In homozygous 4.1(-) HE, the primary absence of protein 4.1 causes a 70% to 90% loss of GPC (approximately 40,000 to 80,000 molecules) and a loss of all p55 molecules. In heterozygous 4.1(-) HE, the reduced amounts of both p55 and GPC were proportional to that of protein 4.1 (Sondag et al.,12 Reid et al.,13 and present data). Therefore, even though the decreased amount of protein 4.1 is still sufficient to saturate both p55 and GPC, for some reason these three proteins remain in a constant ratio in 4.1(-)-heterozygotes.

![Fig 1. Protein 4.1 and p55 in different types of genetically abnormal red cell. Gels were stained with Coomassie blue. Lane a, control. Lane b, homozygous 4.1(-) HE (individual KB) (individual SA II.9 gave an identical pattern; not shown). Lanes c and d, Leach HE (individuals MWB and LN, respectively). Lane e, heterozygous 4.1(-) HE (individual DU II.1) (individual CA II.1 gave an identical pattern; not shown). Lane f, Ge(-) individual O11.DO. The reduction or absence of proteins (protein 4.1 or the 55-Kd protein) are indicated as or , respectively.](image-url)
Proposed models. Based on the above results, we suggest that protein 4.1, p55, and GPC interact with each other. One possibility would be that p55 interacts directly with protein 4.1 and GPC, respectively, and, therefore, mediates the interaction between these two proteins (Fig 3a). That p55 may interact with protein 4.1 is supported by a number of observations. Similar to protein 4.1, p55 remains associated with inside-out vesicles (IOVs) after low ionic strength extraction and is partly extracted by alkaline treatment of IOVs.\(^5\) It is solubilized from erythrocyte membranes with Triton X-100/KCl solutions (Sigma).\(^5\) A significant amount of p55 is extracted along with protein 4.1 from membranes using high salt concentrations.\(^5,3\) Trace amounts of p55 are detectable even when protein 4.1 in high salt extracts is further purified on an anion exchange column (Chishti et al, unpublished results). Purified p55 directly associates with protein 4.1 in blot overlay assays in the absence of GPC (Chishti et al, unpublished results). We cannot rule out that some p55 is present in intact RBCs from individuals with homozygous 4.1(−) HE or Leach HE but would be removed subsequently during preparation of ghosts in the low ionic strength buffer.

In contrast, we have no evidence for a direct interaction of p55 with GPC. Therefore, an alternative possibility would be that p55 interacts only with protein 4.1. Subsequently, the protein-4.1–p55 complex would bind to GPC through protein 4.1 only (Fig 3b). This second model would be consistent with in vitro studies that suggest direct interaction between GPC and protein 4.1.\(^30\) However, one cannot rule out the possibility that the purified protein 4.1 used in these studies contained trace amounts of p55. Although we await the results of direct binding studies in solution and reconstitution assays, the presently available data are consistent with the view that p55 is involved in the interaction of protein 4.1 with GPC. At this stage, we have no evidence that p55 interferes in the binding of protein 4.1 to glycoporphin A and band 3.

Purified protein 4.1 reconstituted to protein–4.1–deficient membranes has been shown to restore membrane stability.\(^13,41\) More recently, the recombinant 10-Kd domain appeared to have the same effect.\(^40\) To account for these results, one may hypothesize that in these experiments protein 4.1 or the recombinant 10-Kd fragment bound only to the spectrin-actin complex, a binding that does not require p55. Similarly in Leach elliptocytes, protein 4.1 (80% of the total stock) would be stabilized through its interaction with the spectrin-actin complex.

The fact that a fraction of GPC was recovered in Triton shells (Sigma) from protein 4.1 reconstituted membranes\(^13\) could be interpreted as evidence against a requirement for p55 in mediating a 4.1–GPC linkage. However, the possibility exists that the preparation of salt-extracted protein 4.1 contained trace amounts of p55, thus allowing protein 4.1 to hook the small quantity of GPC present in the membranes (initially deficient in protein 4.1) used in these experiments. Only reconstitution experiments with recombinant protein 4.1 or 30 Kd domain will definitely address the question.

Human RBC p55,\(^5,3\) protein 4.1,\(^32\) and glycoporphin C\(^17\) are widely distributed proteins. If these three proteins are

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### Table 1. Quantitative Data on Protein 4.1 and the 55-Kd Protein

<table>
<thead>
<tr>
<th>Person</th>
<th>55/4.1</th>
<th>4.1/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leach elliptocytosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MWB</td>
<td>0⁺,†</td>
<td>12.8⁺ (−21%)</td>
</tr>
<tr>
<td>LN</td>
<td>0⁺,†</td>
<td>12.5⁺ (−23%)</td>
</tr>
<tr>
<td>Heterozygous 4.1(−)HE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA, II, 1⁺,†</td>
<td>13⁺</td>
<td>10.8⁺ (−33%)</td>
</tr>
<tr>
<td>DU, II, 1⁺,†</td>
<td>14⁺</td>
<td>12.2⁺ (−25%)</td>
</tr>
<tr>
<td>Controls</td>
<td>12 ± 2⁺</td>
<td>16.25 ± 1.30⁺</td>
</tr>
</tbody>
</table>

*Significant reduction (<m = 2SD; P < .01).† Corrected ratio following subtraction of the background as measured in homozygous 4.1(−) HE individual KB (protein 4.1 absent) (not shown).§ From Feddal et al.\(^16\); however, the values given were re-determined for the present study.

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Note: Ratios are given as percentages. SDS-PAGE was performed (9% acrylamide concentration) and scanned (570 nm) after Coomassie blue staining as previously described or referred to.\(^16,16\)
involved in a complex in the erythrocyte, the same might be true in other cell types. The role of the N-terminal domain, the SH3 motif, and the guanylate kinase domain of p55 is unknown, nor do we know about the regulatory pathways that involve phosphorylation and palmitoylation of this protein. This study provides p55 with a possible function in cross-bridging the skeletal network to the plasma membrane in erythroid and, perhaps, some nonerythroid cells.

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Evidence that red blood cell protein p55 may participate in the skeleton-membrane linkage that involves protein 4.1 and glycophorin C