Molecular Basis of Spectrin Deficiency in Hereditary Pyropoikilocytosis

By Manjit Hanspal, Jatinder S. Hanspal, Kenneth E. Sahr, Eitan Fibach, James Nachman, and Jiri Palek

Hereditary pyropoikilocytosis (HPP) is a recessively inherited hemolytic anemia characterized by severe poikilocytosis and red blood cell fragmentation. HPP red blood cells are partially deficient in spectrin and contain a mutant α or β-spectrin that is defective in terms of spectrin self-association. Although the nature of the latter defect has been studied in considerable detail and many mutations of α-spectrin and β-spectrin have been identified, the molecular basis of spectrin deficiency is unknown. Here we report two mechanisms underlying spectrin deficiency in HPP. The first mechanism involves a thalassemia-like defect characterized by a reduced synthesis of α-spectrin as shown by studies involving synthesis of spectrin in two unrelated HPP probands and their parents: One parent carries the elliptocytogenic spectrin mutation, whereas the other parent is fully asymptomatic. Peripheral blood monocellular cells as a source of erythroid burst-forming unit (BFUs) were cultured in a two-phase liquid culture system that gives rise to terminally differentiated erythroblasts. Pulse-labeling studies of an equal number of erythroblasts of morphologically identical maturity showed that the synthesis of α-spectrin as well as the mRNA levels as measured by the competitive polymerase chain reaction (PCR) method are markedly reduced in the presumed asymptomatic carriers and the HPP probands. In contrast, the synthesis and mRNA levels of β-spectrin were normal. These results constitute a direct demonstration of an α-spectrin synthetic defect in a subset of asymptomatic carriers of HPP and HPP probands. The second mechanism underlying spectrin deficiency involves increased degradation of mutant spectrin before its assembly on the membrane. This is evidenced by pulse labeling studies of erythroblasts from a patient with HPP associated with a homozygous state for spectrin α/β mutation (leu-pro mutation at AA 207 of α-spectrin). These studies showed that although spectrin is synthesized in the cytosol in normal amounts, the rate of turnover of α-spectrin is faster resulting in about 40% to 50% reduced assembly of α-spectrin and β-spectrin on the membrane. Thus, spectrin deficiency in this case is at least in part caused by increased susceptibility of the mutant spectrin to degradation before its assembly on the membrane. We conclude that at least two separate mechanisms underlie the molecular basis of spectrin deficiency in HPP.

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mutant spectrin leading to a premature degradation of this protein before its assembly on the membrane.

To determine the molecular basis of spectrin deficiency in these two subsets of patients with HPP, we have studied the synthesis, assembly, and turnover of α-spectrin and β-spectrin in HPP and control erythroblasts isolated from two-phase liquid cultures of peripheral blood (PB) mononuclear cells. We provide evidence that the spectrin deficiency in HPP is a consequence of at least two distinct mechanisms proposed above. The first underlying mechanism involves reduced synthesis and mRNA content of α spectrin in two unrelated presumed asymptomatic carriers and HPP probands. The second mechanism underlying spectrin deficiency in HPP patients who are either homozygous or doubly heterozygous for one or two spectrin mutations, respectively, involves increased degradation of the mutant spectrin before its assembly on the membrane.

MATERIALS AND METHODS

Subjects. The first subject, S.B., is a 37-year-old white woman with severe HPP requiring splenectomy. Her blood smear showed marked poikilocytes, microspherocytes, and RBC fragmentation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting analysis showed the presence of truncated α-spectrin in the RBC membranes with an approximate molecular weight of 235 Kd that migrated just below the intact α-spectrin chain on SDS gels. Furthermore, the spectrin to band 3 ratio was markedly decreased from 1.0 to 0.68 indicating a moderately severe deficiency of spectrin. The amount of the truncated α-spectrin was approximately 10% of intact α-spectrin chain. There was an increased proportion of spectrin dimers (29% as compared with 4% in controls) in the crude 4°C spectrin extract as well as an increased amount of δG Polypeptide and a decreased amount of the normal 80-Kd fragment after two-dimensional gel analysis of a partial tryptic digest of spectrin. The underlying molecular defect involves an insertion within the α-spectrin gene leading to an in-frame deletion of exon 5 at the cDNA level.

The mother of subject S.B., V.B., carries the spectrin δG mutation, whereas the father, C.B., is hematologically and biochemically normal (with normal amounts of spectrin, 80-Kd fragment, and spectrin dimers in the crude 4°C spectrin extract).

The second subject, B.S., is a 13-year-old white girl with severe HPP requiring splenectomy at the age of 4. The PB film showed the presence of microspherocytes, poikilocytes, and RBC fragments. Biochemical analysis showed an increased proportion of spectrin dimers in the crude 4°C extract (37% as compared with 5% in controls). The spectrin to band 3 ratio was 0.76 as compared with control values of 1.0, indicating a partial deficiency of spectrin. Structural studies indicated that the subject is heterozygous for spectrin δG mutation with an increased amount of the δG fragment and a markedly decreased amount of the normal 80-Kd fragment in the limited tryptic digest of spectrin. Her father, P.S., carries the spectrin δG mutation, whereas the mother, K.S., is hematologically and biochemically normal. The underlying defect in subjects carrying the spectrin δG mutation involves a point mutation causing arginine to histidine (CGT-CAT) substitution at codon 28 of α-spectrin.

The clinical and biochemical data on the third patient, T.N., HPP who is a homozygote for spectrin δG mutation, has been reported previously by Coetzee et al.13 The subject is a 21-year-old black man with severe inherited hemolytic anemia fulfilling the diagnostic criteria of HPP. The underlying defect has recently been identified as a point mutation causing the replacement of leucine by proline at amino acid 207 in the α-spectrin chain.14 His mother is an asymptomatic carrier of αδG466 carrier and his father is not available for studies. Venous blood was collected from the subjects and normal control donors in tubes or bags containing acid citrate dextrose as anticoagulant. The samples were cooled and transported overnight on ice in insulated containers to Boston, where they were analyzed immediately.

Two-phase liquid culture system for the maturation of erythroid progenitors. PB was obtained from healthy donors and patients, and mononuclear cells were separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient density centrifugation. The mononuclear cells were collected and cultured using a slight modification of the two-phase liquid culture system described by Fibach et al15 and Wada et al.16 The mononuclear cells were cultured in the first phase at a density of 10^6/mL in alpha minimum essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Hy Clone, Logan, UT) and 10% conditioned medium collected from cultures of the 5637 bladder carcinoma cell line.17 After a 1-week incubation at 37°C in an atmosphere of 5% CO₂, the nonadherent cells were harvested and treated by the carboxyl iron method18 to remove phagocytic cells. The floating, nonphagocytic cells were recultured in alpha medium containing 30% FCS, 1% deionized bovine serum albumin (BSA), 10⁻³ mol/L 2-mercaptoethanol, 1.5 mmol/L glutamine, 10⁻⁴ mol/L dexamethasone, cyclosporine A, and 1 U/mL human recombinant erythropoietin (generously provided by Ortho Biotec, Raritan, NJ). These secondary cultures were incubated at 37°C in a humidified atmosphere containing 5% O₂ and 5% CO₂. After 4 days in the second phase, when clusters of proerythroblasts start to appear, the cells were harvested (the medium was saved), suspended in 2 mL of the culture medium, layered on a 2-mL solution of 45% Percoll (Pharmacia) in phosphate-buffered saline (PBS) (final density = 1.0585 g/mL), and centrifuged at 2000 rpm for 5 minutes at room temperature. The upper layer containing predominantly proerythroblasts were collected, washed, resuspended in the original culture medium, and incubation continued at 37°C in an atmosphere of 5% O₂ and 5% CO₂. The pellet containing greater than 90% of the lymphocytes was discarded. After 1 week in the second phase, viable cells were counted by trypan blue exclusion. Hemoglobin-containing cells were scored with use of the acetic acid–benzidine peroxidase reaction19 and cellular morphology assessed by preparing cytosin slides stained with Wright-Giemsa.

Pulse labeling of cells with [³⁵S]methionine. Late erythroblasts isolated from liquid cultures were washed twice in MEM without methionine and resuspended in 10 mL of the same medium containing 10% FCS prewarmed to 37°C. The cells were then labeled with [³⁵S]methionine (30 μCi/mL, 1000 Ci/mmol; ICN Biomedicals, Irvine, CA) for different lengths of time. For the pulse-chase experiments, further incorporation of [³⁵S]methionine was stopped by the addition of unlabeled methionine (0.4 mmol/L) and the incubation was then continued for different time periods. At the end of the labeling period, 10 vol of 155 mmol/L choline chloride and 5 mmol/L HEPES, pH 7.1, were added and the cells were harvested by centrifugation.

Cell fractionation and immunoprecipitation. [³⁵S]Methionine-labeled cells were treated with disopropyl fluorophosphosphate (Sigma Chemical Co, St Louis, MO), lysed in 4 vol of lysis buffer (150 mmol/L NaCl, 10 mmol/L TRIS-HCl, pH 7.2, 5 mmol/L MgCl₂, 2 mmol/L EDTA, 0.25 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulphonyl fluoride, 1 mmol/L leupeptin, 10 μg/mL aprotonin, and 1% Triton X-100), and separated into soluble and insoluble fractions, as described earlier20 to measure rates of synthesis and assembly into the membrane skeleton, respectively. Spectrin was immunoprecipitated from these fractions using affinity-purified antispectrin antibodies by the method described previously.20
RNA isolation and quantitation of α-spectrin and β-spectrin mRNAs by competitive PCR. Total cellular RNA was isolated from morphologically identical control and HPP erythroblasts by the guanidinium/cesium chloride method. Total RNA was reverse transcribed into single-stranded cDNA using the antisense primers αSp-B and βSp-B (Fig 1). The reverse transcribed cDNA was co-amplified with a dilution series of either a 13-kb genomic α-spectrin plasmid DNA or a 550-bp genomic β-spectrin plasmid DNA using oligonucleotide primers αSp-A and αSp-B in exons 9 and 10 of the α-spectrin gene or a 550-bp genomic β-spectrin plasmid DNA using oligonucleotide primers βSp-A and βSp-B in exons X and Y of the β-spectrin gene. [32P] deoxyctydidine triphosphate (dCTP) was included in the polymerase chain reaction (PCR) and 45 cycles of amplification were performed with each cycle consisting of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C. After amplification, the samples were examined on an agarose gel containing ethidium bromide, followed by autoradiography. The resulting autoradiograms were scanned and the area under each peak measured. The amount of gDNA was multiplied by the ratio of cDNA bp per gDNA bp to correct for increased label incorporation per mole by the larger fragment. gDNA/cDNA was then plotted as a function of the amount of known competitive gDNA. The point where this ratio is 1 represents the concentration of cDNA in the unknown.

The β-spectrin oligonucleotide primers (βSp-A and βSp-B) are approximately 10-fold less efficient than the α-spectrin primers as determined by PCR amplification of 1 μg each of genomic α-spectrin and β-spectrin plasmid DNAs under identical conditions (data not shown). Accordingly, the β-spectrin mRNA levels are multiplied by a factor of 10.

Sequencing and single-strand conformation polymorphism (SSCP) analysis of α-spectrin cDNA. Total cellular RNA was reverse transcribed using random hexanucleotides (New England Biolabs, Beverly, MA) and PCR amplified with primers 404 (5'-TGAGGAAAGGAGCCTTGA-3'; bases 5676 to 5686) and 405 (5'-GGATGTCTCTCCTCTGTGCA-3'; bases 5839 to 5820) using the Perkin-Elmer (Norwalk, CT) GeneAmp PCR Reagents kit (45 cycles, 1 minute at 94°C, 1 minute at 52°C, 30 seconds at 72°C). An aliquot of the PCR product was cloned into plasmid pCR 1000 using the TA cloning kit (Invitrogen, San Diego, CA) and inserts were sequenced with the Sequenase version 2 sequencing kit (US Biochemical Corp, Cleveland, OH).

For single-strand conformation polymorphism (SSCP) analysis, reverse transcribed cDNA was PCR amplified in the presence of [32P]dCTP using α-spectrin primers 404 and 405. The PCR product was heat denatured and separated by non-denaturing electrophoresis in Mutation Detection Enhancement (MDE) gel (AT Biochem, Inc, Malvern, PA), followed by autoradiography.

RESULTS

In vitro cultured human erythroblasts replicate skeletal protein synthesis of explanted human erythroblasts. On day 12 or 13 of the second phase of the liquid cultures, greater than 90% of the cells are benzidine positive and appear to be at the late erythroblast (orthochromatophilic normoblast) stage as shown by Wright-Giemsa staining of the cytopsin slides (Fig 2). To ascertain that the two-phase liquid culture system used here to study spectrin synthesis reflects biosynthetic events as they occur in vivo, we have compared spectrin synthesis in the in vitro cultured erythroblasts with that in explanted human bone marrow (BM) erythroblasts and PB reticulocytes. The synthesis of α-spectrin and β-spectrin polypeptides was measured in an equal number of cells. The amounts of newly synthesized α-spectrin and β-spectrin polypeptides in the cultured erythroblasts and the α-spectrin to β-spectrin synthetic ratios were similar to those in the explanted BM erythroblasts and in reticulocytes when corrected for cell maturity based on the total cellular RNA content (data not shown), which suggests that the in vitro culture conditions accurately reflect the synthetic events in explanted BM erythroblasts and thus are likely to be representative of biosynthetic events in vivo.

A subset of patients with HPP carry a defect involving reduced expression and synthesis of α-spectrin. As outlined above, in the most common type of HPP, the probands appear to inherit two defects: (1) an elliptocytogenic spectrin mutation inherited from one of the parents who is either asymptomatic or has a mild HE, and (2) another defect that augments the quantitative expression of the mutant spectrin in the cells producing a concomitant spectrin deficiency. Here we tested the hypothesis that this second defect represents a “thalassemia-like” defect involving reduced spectrin synthesis. To address this possibility, we conducted pulse-labeling studies of an equal number of late erythroblasts isolated from cultures that were morphologically identical in terms of erythroblast stage. These studies showed that in the normal control and one parent who is an HE carrier, α-spectrin was synthesized in about threefold excess over β-spectrin consistent with previous reports.

In a striking contrast, in the asymptomatic carrier and the HPP proband, the synthesis of α-spectrin was markedly re-
A SYNTHETIC DEFECT OF \( \alpha \)-SPECTRIN IN HPP

**A**

![Graph showing synthesis and assembly of spectrin](image)

**B**

![Graph showing synthesis and assembly of spectrin](image)

A SYNTHETIC DEFECT OF \( \alpha \)-SPECTRIN IN HPP

Reduced, with an \( \alpha \)-spectrin to \( \beta \)-spectrin synthetic ratio of 1.4 and 1.0, respectively (Fig 3A). Because the synthesis of \( \beta \)-spectrin was within a normal range in all the subjects, these results clearly show that the synthesis of \( \alpha \)-spectrin is markedly reduced in both the carrier and the proband.

Next, we examined the assembly of newly synthesized spectrin polypeptides into the membrane skeleton. Measurements of the assembly of \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides into the membrane skeleton fraction showed that in the asymptomatic carrier, the amounts of both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides incorporated into the membrane skeleton were normal in spite of the above noted decrease in \( \alpha \)-spectrin synthesis. In contrast, in the HPP proband, the assembly of \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides in the membrane skeleton was reduced by 30% to 40% (Fig 3B). To further correct for the possible differences in the maturity of erythroblasts between control and patient, we also measured the assembly of newly synthesized ankyrin into the membrane skeleton, as described earlier.

Because the synthesis of \( \alpha \)-spectrin is reduced in both the asymptomatic carrier and the HPP proband, but the assembly of spectrin on the RBC membrane is reduced only in the proband, we examined the possibility that the mutant spectrin present in the proband was unstable. Indeed, pulse-chase analysis showed that the turnover of the mutant spectrin present in the proband and the HE parent was faster than that of the normal spectrin: The half-life of newly synthesized \( \alpha \)-spectrin was 233 minutes in the asymptomatic carrier and the HPP proband, reduced \( \alpha \)-spectrin synthesis together with the instability of the mutant spectrin causes a partial spectrin deficiency.

To further elucidate the mechanism of reduced \( \alpha \)-spectrin synthesis, we examined \( \alpha \)-spectrin mRNA levels in the asymptomatic carrier and the HPP proband. Measurement of \( \alpha \)-spectrin mRNA levels by competitive PCR technique showed that \( \alpha \)-spectrin mRNA levels were markedly reduced in both the asymptomatic carrier and the HPP proband as compared with the normal control (Fig 4). To assure that this difference was not caused by possible differences in cell age between cultured control and patient erythroblasts, we measured \( \beta \)-spectrin mRNA levels in these individuals and the ratio of \( \alpha \)-spectrin to \( \beta \)-spectrin mRNA levels was compared. As shown in Table 1, the \( \alpha \)-spectrin to \( \beta \)-spectrin mRNA ratio was markedly reduced in both the asymptomatic carrier and the proband. The \( \alpha \)-spectrin and \( \beta \)-spectrin mRNA levels in the HE parent were in the normal range (data not shown). These results are complementary to the synthetic studies described above suggesting that the synthesis of \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides in erythroblasts is determined by their mRNA levels.

Although the molecular basis of reduced \( \alpha \)-spectrin mRNA content is unknown, several possible defects at the DNA level could cause this phenotype (see Discussion section). Recently, a common, asymptomatic spectrin polymorphism (\( \alpha^{174} \)) has been found in association with a low expression of \( \alpha \)-spectrin. Therefore, we have asked whether or not this polymorphism is present in our subjects with HE and HPP. We have PCR amplified a cDNA fragment of 173 bp from the subjects C.B., V.B., S.B., and C.B. using primers specific for \( \alpha \)-spectrin. The PCR products were sub-
cloned, and multiple clones from each individual were pooled and sequenced. We found that both the proband (S.B) and the asymptomatic parent (C.B) were heterozygous for this polymorphism (AA 1857 leu-val, CTA-GTA substitution), whereas the other parent (V.B) who came the elliptocytogenic mutation was normal (data not shown). These results show that in this particular situation the polymorphism is coinherited with the α-spectrin synthetic defect. However, further studies are required to determine whether or not this polymorphism is linked with the α-spectrin synthetic defect.

In the second HPP proband who carries the spectrin α1/74 mutation characterized by Arg-His (CGT-CAT) substitution at codon 28 of α-spectrin (data not shown), we have likewise postulated the presence of a second defect involving a reduced synthesis of α-spectrin. To test this, we conducted pulse-labeling studies similar to those described above for the first patient (S.B.). Indeed, we found similar results: The synthesis of α-spectrin was markedly reduced in both the asymptomatic carrier and the HPP proband, whereas it was normal in the HE parent. Likewise, the assembly of the newly synthesized spectrin polypeptides into the membrane skeleton was normal in both parents but was reduced by about 40% in the HPP proband (Fig 5).

Next, we examined the α-spectrin mRNA levels in the HPP proband. In this case, because the HE mutation altered a restriction enzyme site (codon 28 Arg-His abolishes an AhaII site9) the mRNA transcripts derived from each of the two spectrin alleles could be distinguished. Therefore, a 400-bp fragment of α-spectrin cDNA containing the mutation was PCR amplified followed by digestion with the enzyme AhaII. The digested PCR product was examined on an agarose gel, transferred to a zeta probe membrane, and hybridized with [32P]-labeled α-spectrin probe. As shown in Fig 6, the proband is heterozygous for the mutant allele that has lost the AhaII site and, hence, remains intact (400-bp

**Table 1. α-Spectrin and β-Spectrin mRNA Levels**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Control</th>
<th>C.B</th>
<th>S.B</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSp mRNA (ng)</td>
<td>10.00</td>
<td>5.60</td>
<td>2.95</td>
</tr>
<tr>
<td>βSp mRNA (ng)</td>
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<td>2.40</td>
<td>2.00</td>
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<tr>
<td>α/β Sp mRNA</td>
<td>4.76</td>
<td>2.33</td>
<td>1.47</td>
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</table>

α-Spectrin and β-spectrin mRNA levels were determined by competitive PCR using total RNA isolated from in vitro cultured control, asymptomatic carrier (C.B.) and the HPP proband (S.B.) erythroblasts.

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**Fig 4.** Quantitation of α-spectrin mRNA by competitive PCR. (A) Various amounts of genomic α-spectrin (1 to 20 ng) competed against a fixed concentration of α-spectrin cDNA in the presence of [32P]dCTP. After PCR amplification, the samples were analyzed on a 2% agarose gel followed by autoradiography. (B) Plot of the ratio of genomic α-spectrin/cDNA after amplification versus genomic DNA added to the original mixture. α-Spectrin mRNA level is markedly reduced in both the asymptomatic carrier and the HPP proband.

**Fig 5.** Synthesis and assembly of spectrin in erythroblasts from control and subjects of the second family. Late erythroblasts were isolated from the presumed asymptomatic carrier (K.S.), the HPP proband (B.S.), and the other parent (P.S.) who is an HE carrier and were metabolically labeled with [35S]methionine for 45 and 90 minutes. Synthesis and assembly of α-spectrin and β-spectrin polypeptides was measured at both time points and the results were linear. Values obtained during the 90-minute labeling are shown here. Experimental details are given in the legend to Fig 3. Because multiple blood samples were not available from the family, this experiment was done once in duplicates with similar results.
In this report, we have defined two mechanisms underlying spectrin deficiency in a subset of patients with HPP. The first mechanism involves a compound heterozygote state for mutant \( \alpha \)-spectrin or \( \beta \)-spectrin and a defect representing reduced synthesis of \( \alpha \)-spectrin. This is evidenced by pulse-labeling studies of in vitro cultured erythroblasts which showed that in contrast to controls, two unrelated HPP probands exhibited a marked decrease in the synthesis and mRNA levels of \( \alpha \)-spectrin. Analysis of the parents of these subjects showed that although the synthesis of \( \alpha \)-spectrin was normal in the parents carrying the elliptocytogenic mutation, the synthesis and mRNA levels of \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides were synthesized in normal amounts. However, the amount of both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides assembled on the HPP erythroblast membrane was reduced to 40% to 50% of the normal values (Fig 7) consistent with reduced steady-state amounts of \( \alpha \)-spectrin and \( \beta \)-spectrin on the HPP erythrocyte membrane.

Because the synthesis of both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides was found normal, whereas their assembly on the membrane was markedly reduced, we explored the possibility that the reduced assembly of spectrin on the membrane was a consequence of accelerated degradation of the spectrin polypeptides before their assembly on the membrane. Indeed, pulse-chase analysis of the newly synthesized \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides showed a marked increase in the rate of turnover of \( \alpha \)-spectrin in the HPP erythroblast cytosol as compared with a normal control. The rate of turnover of \( \beta \)-spectrin was also increased in HPP erythroblasts as compared with control erythroblasts. However, the latter could be caused by increased formation of \( \beta \)-spectrin homodimers (in the absence of intact \( \alpha \)-spectrin) that exhibit accelerated degradation. In the proband’s mother who is an asymptomatic spectrin \( \alpha^{46/41} \) carrier, we also found an increase in the rates of turnover of \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides (data not shown). However, the synthesis and assembly of spectrin on the membrane was normal suggesting that the increased degradation of the mutant spectrin before its assembly on the membrane leads to spectrin deficiency in the homozygote offspring but is asymptomatic in a simple heterozygote.

**DISCUSSION**

In this report, we have defined two mechanisms underlying spectrin deficiency in a subset of patients with HPP. In contrast to the most characteristic pattern of HPP inheritance outlined in the preceding paragraph, some subjects with HPP are either homozygotes or double heterozygotes for two elliptocytogenic mutations. To elucidate the molecular basis of spectrin deficiency in this subset of patients, we studied synthesis of spectrin in morphologically identical erythroblasts of a control subject and a patient with HPP homozygous for spectrin \( \alpha^{46/41} \) mutation (leu-pro mutation at amino acid 207 of \( \alpha \)-spectrin). These studies showed that in this subject, both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides were synthesized in normal amounts. However, the amount of both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides assembled on the HPP erythroblast membrane was reduced to 40% to 50% of the normal values (Fig 7) consistent with reduced steady-state amounts of \( \alpha \)-spectrin and \( \beta \)-spectrin on the HPP erythrocyte membrane.

Increased turnover of \( \alpha \)-spectrin before its assembly on the membrane leads to a partial spectrin deficiency in the second subset of patients with HPP. In contrast to the most characteristic pattern of HPP inheritance outlined in the preceding paragraph, some subjects with HPP are either homozygotes or double heterozygotes for two elliptocytogenic mutations. To elucidate the molecular basis of spectrin deficiency in this subset of patients, we studied synthesis of spectrin in morphologically identical erythroblasts of a control subject and a patient with HPP homozygous for spectrin \( \alpha^{46/41} \) mutation (leu-pro mutation at amino acid 207 of \( \alpha \)-spectrin). These studies showed that in this subject, both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides were synthesized in normal amounts. However, the amount of both \( \alpha \)-spectrin and \( \beta \)-spectrin polypeptides assembled on the HPP erythroblast membrane was reduced to 40% to 50% of the normal values (Fig 7) consistent with reduced steady-state amounts of \( \alpha \)-spectrin and \( \beta \)-spectrin on the HPP erythrocyte membrane.

**DISCUSSION**

In this report, we have defined two mechanisms underlying spectrin deficiency in a subset of patients with HPP. The first mechanism involves a compound heterozygote state for mutant \( \alpha \)-spectrin or \( \beta \)-spectrin and a defect representing reduced synthesis of \( \alpha \)-spectrin. This is evidenced by pulse-labeling studies of in vitro cultured erythroblasts which showed that in contrast to controls, two unrelated HPP probands exhibited a marked decrease in the synthesis and mRNA levels of \( \alpha \)-spectrin. Analysis of the parents of these subjects showed that although the synthesis of \( \alpha \)-spectrin was normal in the parents carrying the elliptocytogenic spectrin mutation, the synthesis and mRNA levels of \( \alpha \)-spectrin were markedly decreased in the parents who are asymptomatic with no detectable abnormalities of spectrin structure and function. The inheritance of this synthetic defect in the asymptomatic carriers is unknown, because we
were unable to study other members of these families. However, based on the fact that the decrease in the synthesis and mRNA level of α-spectrin is approximately 50%, it is likely to be inherited as a heterozygous defect. Moreover, if the carriers were homozygous for this defect, the reduction in spectrin synthesis would be expected to be greater in the parents carrying two biosynthetically defective alleles than in the probands carrying one such allele, which is not the case.

The assembly of the newly synthesized α-spectrin and β-spectrin polypeptides into the membrane skeleton was normal in the asymptomatic parent but was reduced by 30% to 40% in the proband who contains a mutant spectrin. In the first patient, the mutant spectrin is truncated because of an in-frame deletion of exon 5 of α-spectrin. Pulse-chase analysis showed that in the HPP proband and the HE parent (both containing the mutant spectrin), newly synthesized spectrin underwent increased degradation before its assembly on the membrane suggesting a possibility that the mutant spectrin is unstable. Moreover, the fact that the steady-state levels of the truncated spectrin are relatively small (approximately 10% of total spectrin) in both the elliptocytic parent and the proband further supports the possibility that the mutant spectrin is unstable. Furthermore, because the truncated spectrin is the result of an in-frame deletion of exon 5, one could anticipate that this deletion will lead to an incorrect phasing of the repeat units, which in turn will cause an accelerated degradation of the mutant spectrin.

This is consistent with recent findings that spectrin polypeptides with incorrectly phased repeating motifs yield unstable structures. Thus, our findings can be interpreted as follows: (1) In a simple heterozygous carrier, in whom one α-spectrin allele is normal, whereas the other allele exhibits reduced synthesis, the reduced α-spectrin synthesis is fully asymptomatic because the net synthesis of α-spectrin remains in excess of β-spectrin; and (2) in a double heterozygote who co-inherited a defect involving a reduced α-spectrin synthesis together with an unstable spectrin mutant, the amount of α-spectrin available for membrane assembly is markedly reduced causing a partial deficiency of spectrin.

Our findings of reduced α-spectrin synthesis in the asymptomatic carrier and the proband are further supported by our data indicating a marked decrease in their α-spectrin mRNA contents. The results presented in this article thus constitute a direct demonstration of an α-spectrin synthetic defect in an asymptomatic carrier of HPP. This synthetic defect, when transmitted to the HPP offspring, increases the quantitative expression of the α-spectrin mutant and causes a concomitant partial spectrin deficiency.

Recently, an asymptomatic spectrin polymorphism (α145) has been described that is characterized by an increased susceptibility to proteolysis of the αIV-αV domain junction as a result of AA 1857 leu-Val substitution. In subjects with HE carrying mutant α-spectrin, the presence of this polymorphism in trans enhances the biochemical, morphologic, and clinical expression of the elliptocytogenic
A SYNTHETIC DEFECT OF α-SPECTRIN IN HPP

spectrin mutation. Therefore, we have examined whether or not this polymorphism is present in our subjects with HE and HPP. We find that both the probands and the asymptomatic parent (of the first patient) carrying the defect of spectrin synthesis are heterozygous for this polymorphism (AA 1857 leu-val substitution), whereas the elliptocytic parent are normal. At this stage, the cause-effect relationship of this polymorphism and the α-spectrin synthetic defect is not known. However, homozygotes for the αV461 defect are known to be asymptomatic and thus are unlikely to exhibit a markedly reduced synthesis of α-spectrin that would be expected to cause a severe spectrin deficiency. The molecular mechanism underlying reduced spectrin mRNA levels and synthesis in the patients reported above is unknown and is currently under study.

The second mechanism underlying spectrin deficiency in a subset of patients with HPP who are either homozygotes or doubly heterozygotes for one or two elliptocytic spectrin mutations appears to involve decreased stability of the mutant spectrin leading to reduced assembly of the mutant spectrin on the RBC membrane. This is suggested by the following findings: (1) in a subject with HPP who is a homozygote for α-spectrin αV461 mutation, the synthesis of α-spectrin and β-spectrin polypeptides was normal but their assembly on the erythroblast membrane was reduced to 40% to 50% of the normal values; and (2) pulse-chase analysis showed that in the HPP erythroblast cytosol, the rate of turnover of newly synthesized α-spectrin was markedly increased. The rate of turnover of β-spectrin was also found to be increased in HPP erythroblasts. The latter result is in context with the previous studies in avian and mammalian (M. Hanspal et al, unpublished observation, 1990) erythrocyte cells that showed that the newly synthesized α-spectrin and β-spectrin chains exist in the cytosol as heterodimers (the homooligomers undergo rapid degradation), and that the assembly of α-spectrin heterodimers is virtually instant. Hence, if α-spectrin is unstable within a given heterodimer, the corresponding β-spectrin chain will also be expected to be unstable, because it cannot maintain its stability without the accompanying α-spectrin chain, and thus resulting in markedly reduced amounts of intact spectrin available for membrane assembly. In addition, it is possible that the markedly increased turnover of mutant spectrin in this patient is caused by an increased production of a protease. The results presented here suggest that the molecular basis of spectrin deficiency in this subject with HPP associated with a homozygous state for spectrin αV461 is at least in part the result of increased degradation of mutant spectrin before its assembly on the membrane.

In addition to the mechanisms discussed above, other possible abnormalities could account for partial spectrin deficiency in a subset of patients with HPP. One such possibility may be related to the stoichiometry of spectrin to ankyrin: In the normal RBC membrane, the spectrin-ankyrin binding stoichiometry is one copy of ankyrin per spectrin tetramer. Consequently, in patients with severely disrupted spectrin self-association and a marked increase in unassembled dimeric spectrin in the membrane, the number of ankyrin binding sites may be insufficient to bind all spectrin dimers to the membrane.

Taken together, we show that the spectrin deficiency in HPP is a consequence of at least two molecular defects. The first defect involves reduced synthesis of α-spectrin that augments the quantitative expression of the mutant spectrin and causes a concomitant partial spectrin deficiency. The second defect involves increased degradation of mutant spectrin before its assembly on the membrane.

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