Identification of the 37-kDa Protein Displaying a Variable Interaction with the Erythroid Cell Membrane as Glyceraldehyde-3-phosphate Dehydrogenase*

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In previous studies from this laboratory we isolated and characterized a 37-kDa protein that was associated with the membrane of erythroid cells. The polypeptide appeared to undergo a lineage-specific alteration in its interaction with the membrane during erythroid development and migrated as a family of isoelectric focusing variants during analyses on two-dimensional gels. We report here that the 37-kDa protein is homologous to the enzyme glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). This conclusion was reached from the results of several experimental approaches comparing the biochemical and genetic properties of the 37-kDa protein (p37) with authentic glyceraldehyde-3-phosphate dehydrogenase. Peptide maps of highly purified p37 and glyceraldehyde-3-phosphate dehydrogenase, generated with Staphylococcus V8 protease, were identical. The nucleotide sequence of a cDNA clone encoding p37 was nearly identical to the published sequence for genes encoding glyceraldehyde-3-phosphate dehydrogenase. These results suggest that the interaction of the enzyme with the red cell membrane is more complex than previously envisioned. The existence of subpopulations of glyceraldehyde-3-phosphate dehydrogenase molecules is envisioned that exhibit different levels of enzyme activity and bind to the red cell membrane with varying affinities.

The enzyme glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) is composed of identical subunits of 37,000 molecular weight (1–3). The enzyme catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate and enzyme activity is regulated in a number of ways. Activity is inhibited competitively or non-competitively by several glycolytic metabolites and other small molecules (4). Enzyme activity may also be regulated through the association of the enzyme with the cell membrane as reported by Tsai et al. (8) who showed that glyceraldehyde-3-phosphate dehydrogenase bound to the red cell membrane is enzymatically inactive. The association of glyceraldehyde-3-phosphate dehydrogenase with the cytoplasmic face of the red cell membrane has been studied extensively (5–8). In red cells, 60-70% of the enzyme activity is membrane bound and constitutes about 5-7% of the total membrane protein (5). A number of agents will elute glyceraldehyde-3-phosphate dehydrogenase from the membrane including physiological concentrations of NaC (9).

Several recent reports have suggested an involvement of membrane-bound glyceraldehyde-3-phosphate dehydrogenase in the formation of specialized membrane structures. Hultine and Pantaloni (10) found the enzyme to play a regulatory role in the bundling/unbundling of microtubules isolated from brain. The regulatory mechanism suggested by their data involves the conversion of enzyme tetramers (which catalyzes the bundling of microtubules) into dimers (which unbundle microtubules) in an ATP-dependent manner. The ATP concentration in the vicinity of the membrane may regulate the tetramer/dimer equilibrium and hence the extent of polymerization of the microtubular network in brain cells. In separate study, Caswell and Corbett (11) found glyceraldehyde-3-phosphate dehydrogenase subunits involved in the assembly of junctional triads from transverse tubules in skeletal muscle cells. Enzyme associated with cytoskeletal elements was enzymatically inactive. In a more recent study Kawamoto and Caswell (12) found glyceraldehyde-3-phosphate dehydrogenase to exhibit a protein kinase-like activity resulting in the phosphorylation of transverse-tubule proteins that may be involved in the assembly of the junctional triads. Their results strongly suggest a physiological role for glyceraldehyde-3-phosphate dehydrogenase in addition to its role in glycolysis, perhaps as a protein kinase.

We have been studying the molecular properties of a 37-kDa protein (p37) associated with the membranes of a variety of hematopoietic cell types (13, 14). Our results suggest that the interaction of p37 with the red cell membrane differs from that of other cell types in that the protein appears to be expressed on the outside of most cell types whereas the polypeptide is restricted to the inner membrane surface in red cells (13, 14). Furthermore, p37 appears to be expressed on the outside of K562 erythroleukemic cells suggesting that the interaction of the polypeptide with the erythroid membrane changes during maturation of the lineage.

In more recent studies (15), we have found that p37 interacts with the red cell membrane through electrostatic interactions that are resistant to disruption by NaCl. In addition, the polypeptide displays microheterogeneity in its migration during isoelectric focusing (15).

Given the similar properties of glyceraldehyde-3-phosphate dehydrogenase and p37 with regard to size and membrane binding, we performed a comparative analysis of these poly-
peptides. Our results conclusively show that p37 is homologous to glyceraldehyde-3-phosphate dehydrogenase. This, coupled with the knowledge obtained from our previous studies, indicates that the binding of the enzyme to the cell membrane is more complex than previously envisioned and suggests that there may be subpopulations of enzyme molecules that bind to the red cell membrane with varying affinities.

MATERIALS AND METHODS

Cell Culture and Biosynthetic Labeling—K562 cells were maintained at 37°C in a humidified atmosphere of 5% CO2, 95% air in RPMI 1640 medium (K. C. Biological, Lenexa, KS) supplemented with 10% heat-inactivated horse serum (HyClone Laboratories, Logan, UT). K562 cells display characteristics of an erythroid progenitor and synthesize a number of erythroid lineage-specific markers (16, 17).

Cells were labeled for 3 h at 37°C in the presence of 25 μCi/ml [35S]methionine (1100 Ci/mmol, New England Nuclear) in methionine-free RPMI 1640 containing 10% dialyzed horse serum. At the end of the labeling period, cells were pelleted by centrifugation at 500 × g and washed twice with RPMI 1640 medium in preparation for lysis with nonionic detergent.

Detergent Lysis and Immunoprecipitation—A detailed description of our immunoprecipitation protocol has been published previously (13, 14). In brief, when labeled cells were immunoprecipitated, they were first solubilized in Triton X-100, and the lysate was clarified by ultracentrifugation. In vitro translation products were also immunoprecipitated in the presence of Triton X-100. Immune complexes were removed from solution with protein A-bearing Staphylococcus aureus bacteria (18), which were then washed repeatedly in buffer containing 0.2% SDS1 immune complexes were eluted into SDS sample buffer (19) during a 5-min incubation in boiling water. The antisera used was raised in rabbits against p37 purified from ghosts as described previously (15).

Polyacrylamide Gel Electrophoresis—One-dimensional SDS gels were run at 650 mA and 12.5% acrylamide and were prepared and run according to Laemmli (19). After electrophoresis, gels were stained with Coomassie Blue. In some cases, destained gels were impregnated with ENHANCE (New England Nuclear). For autoradiography, gels were dried and exposed to preflushed x-ray film (Kodak XAR) for 5–10 days according to the procedures of Bonner and Laskay (21).

One-dimensional Peptide Mapping—One-dimensional peptide mapping was performed as described by Cleveland et al. (22). The polypeptide to be mapped (either unlabeled or labeled with [35S] methionine) were excised using a scalpel from SDS-PAGE gels that had been briefly stained with Coomassie Blue and then destained with H2O at 4°C to visualize the band.

Samples excised from SDS gels were digested with 5 μg of S. aureus V8 protease (Boehringer Mannheim) as described by Cleveland et al. (22). Peptides generated by V8 digestion were electrophoresed in 15% polyacrylamide gels which were stained with either Coomassie Blue or silver. In cases where labeled polypeptides were mapped with V8, gels were impregnated with ENHANCE (New England Nuclear) and exposed to x-ray film as described above.

Screening the λ gt11 cDNA Library with anti-p37 Antibodies—A λ gt11 cDNA library constructed with mRNA extracted from SV40-transformed human fibroblasts using published techniques (23) was obtained from Dr. B. Wold (California Institute of Technology). The library was alkaline-labeled at a density of approximately 1000–1500 plaques/90-mm Petri dish on a lawn of Escherichia coli Y1090 cells and allowed to incubate at 42°C for 3 h. The plates were then shifted to 37°C and incubated overnight for plaque development. Plaques were overlaid the next morning with an 85-mm nitrocellulose filter impregnated with 0.2% methylmethacrylate and then incubated for a further 6 h at 37°C to induce expression of the cloned polypeptide product fused to β-galactosidase. Filters were then lifted from the plates, blocked for 30 min with phosphate-buffered saline containing 2% bovine serum albumin and ovalbumin and 0.02% Tween 20, and then incubated with antiserum raised against p37 purified from red cell ghosts (15) and absorbed against an equal volume of Y1090 E. coli cells. Following incubation with antiserum, filters were washed with phosphate-buffered saline containing 1 mg/ ml each of bovine serum albumin and ovalbumin and 0.02% Tween 20 and then were incubated with 50,000 cpm/ml 125I-Protein A (specific activity, 2 × 108 cpm/μg) for 30 min at room temperature. Filters were washed and exposed to x-ray film for 24–48 h. Plaques reacting with the antiserum were plaque purified several times before large scale production of phage DNA for further analysis.

Restriction Mapping and DNA Sequencing—Restriction mapping was performed with various enzymes using instructions of the supplier (Bethesda Research Laboratories). Restriction digests were analyzed on 0.8–1.0% agarose gels, and DNA fragments were stained with ethidium bromide. DNA sequencing was performed according to the procedures of Maxam and Gilbert (28).

RESULTS

We purified p37 from membranes of red cells and K562 cells (15) using techniques published by others for the purification of a class of 37-kDa proteins that are phosphorylation targets of retroviral protein kinases (29, 30). Fractions of p37 purified from the red cell membrane to about 80% homogeneity were assayed for glyceraldehyde-3-phosphate dehydrogenase activity using standard techniques (31). Activity was detectable (not shown); however, the specific activity of the enzyme in these fractions was consistently reduced to 5–10% of that reported for glyceraldehyde-3-phosphate dehydrogenase isolated from a number of sources, including human red cells (4). We believe the reduction in enzyme specific activity accompanies the purification procedure either due to a depletion of NAD+ from the enzyme or to the concentration-dependent dissociation of active enzyme tetramers into inactive dimers and monomers (41).

We have previously compared by limited peptide mapping p37 polypeptides immunoprecipitated from in vitro translations of K562 mRNA or from nonionic detergent lysates of K562 cells (14) and p37 purified from red cell ghosts (15). In

FIG. 1. V8 peptide mapping of p37 from various sources and authentic glyceraldehyde-3-phosphate dehydrogenase. Polypeptides to be mapped were first purified either biochemically or immunologically by immunoprecipitation and electrophoresed by SDS-PAGE. The desired bands were excised from the gel with a scalpel and then digested with 5 μg of Staphylococcus V8 protease using the Cleveland technique as described under "Materials and Methods." Lane A, peptide map of glyceraldehyde-3-phosphate dehydrogenase purchased commercially (Sigma) and then further purified by blue Sepharose chromatography (10, 32) and SDS-PAGE. Lane B, peptide map of p37 purified from red cell ghosts. Lane C, peptide map of an unrelated 20-kDa polypeptide. Lane D, peptide map of an autodigest of V8 protease. Lane E, peptide map of p37 immunoprecipitated from [35S]methionine-labeled K562 cells. (This is an autoradiographic exposure.) Arrowheads in the margin reflect the migration of molecular weight standards representing (from top to bottom): 45, 26, 14.3, and 6.2 kDa.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
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all cases homology was found. V8 peptide maps of p37 purified from ghosts or K562 cells were compared to V8 maps of glyceraldehyde-3-phosphate dehydrogenase purchased commercially and purified further by affinity chromatography on Blue Sepharose (10, 32) and SDS–PAGE. As shown in Fig. 1, the V8 peptide maps indicate homology between p37 isolated from red cells or K562 cells and glyceraldehyde-3-phosphate dehydrogenase purchased commercially.

In a separate approach, the λ gt11 cloning system was used in conjunction with the high titered antiserum to p37 to obtain a cDNA clone encoding p37. A recombinant phage was identified that synthesized an immunologically recognizable polypeptide and carried a cDNA insert of 1204 base pairs. The DNA was extracted from this phage, coupled to nitrocellulose, and used to select its complementary mRNA. In vitro translation of the hybridized mRNA followed by immunoprecipitation with anti-p37 antiserum confirmed that the cDNA clone encoded a polypeptide of 37 kDa (Fig. 2). To ensure that the 37-kDa polypeptide encoded in the recombinant λ phage was the same polypeptide we have been studying, V8 protease mapping was performed on the two polypeptides. As shown in Fig. 3, the peptide maps of the two polypeptides are homologous.

Restriction analysis of the cDNA insert (Fig. 4) yielded a map with striking homology to maps published for clones encoding glyceraldehyde-3-phosphate dehydrogenase (33, 34). The complete nucleotide sequence of the p37 cDNA insert was determined and found to encode glyceraldehyde-3-phosphate dehydrogenase (Fig. 5).

Our sequence differs in a minimum number of positions from the nucleotide sequences of glyceraldehyde-3-phosphate dehydrogenase determined by other investigators (33, 34) (Fig. 5).

DISCUSSION

The role of glyceraldehyde-3-phosphate dehydrogenase during glycolysis is to catalyze the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate which is then con-
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The nucleotide sequence of the cDNA insert in the λ clone was sequenced by the Maxam and Gilbert technique (28) and is listed on the line labeled 1 in the figure. The published sequences for encoding glyceraldehyde-3-phosphate dehydrogenase genes according to Hanser and Mandel (33) and Tao et al. (34) are listed on lines 2 and 3, respectively, for comparison. Positions of homology in the latter two sequences are indicated with dots whereas differences are indicated with the appropriate nucleotide. The initiation codon, ATG, is indicated in the sequence underlined and labeled with met, and the stop codon is underlined and labeled with Stop.

FIG. 5. Nucleotide sequence of the cDNA insert in the λ clone and comparison with sequences published for glyceraldehyde-3-phosphate dehydrogenase by others. The nucleotide sequence of the cDNA insert in the λ clone was sequenced by the Maxam and Gilbert technique (28) and is listed on the line labeled 1
sequence of this clone was compared to other glyceraldehyde-3-phosphate dehydrogenase sequences that have been reported (33, 34, 36–38), there was little difference in the coding region. Furthermore, the nucleotide substitutions found in the coding region do not have an effect on the amino acid sequence of the polypeptide.

Several investigators have questioned the existence of multiple genes encoding glyceraldehyde-3-phosphate dehydrogenase since it is known that there are numerous homologous gene sequences scattered throughout the genome (33, 34–36). In cases where these “extra” sequences have been analyzed, they appear to represent noncoding pseudogenes (33, 34) lending support for the notion that there is only one functional gene for the enzyme. The data presented here also support this conclusion since the coding sequence is so highly homologous to that reported by others. It should be noted, however, that in the study of Tso et al. (34) at least one of these presumably noncoding sequences was found represented in a cDNA library indicating the presumed pseudogene was transcribed, perhaps into a nontranslated mRNA molecule.

Data from several groups have supported the notion that there are multiple genes encoding glyceraldehyde-3-phosphate dehydrogenase genes based upon the detection of isoelectric focusing variants of the enzyme (39, 40). Edwards et al. (39) suggested the possibility that the isoelectric focusing variants represented post-translationally modified forms of a single gene product, perhaps through the age-dependent conversion of glutamines and asparagines to their respective deaminated forms. It is, therefore, still unclear whether there is a single or multiple functional genes encoding glyceraldehyde-3-phosphate dehydrogenase.

REFERENCES
20. Deleted in proof