A structural model of human erythrocyte band 2.1: Alignment of chemical and functional domains

(syndeins/ankyrin/spectrin/band 3/proteolytic digestion)

REIDAR WALLIN, ELSIE N. CULP, DIANE B. COLEMAN, AND STEVEN R. GOODMAN*

Department of Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033

Communicated by Charles R. Park, March 15, 1984

ABSTRACT Protein 2.1 is a 210-kilodalton protein that connects erythrocyte spectrin to the NH2-terminal cytoplasmic domain of band 3 and thereby functions as the essential linkage between the membrane skeleton and the bilayer. We cleaved this protein into specific chemical domains by limited digestion with trypsin and α-chymotrypsin at 0°C. Intermediate-sized peptides were separated by two-dimensional isoelectric focusing/NaDodSO4/polyacrylamide gel electrophoresis and characterized by high resolution peptide mapping. We have established a provisional structural model of protein 2.1 by comparing the peptide maps of these chemical domains to maps obtained from larger overlapping chromotryptic fragments as well as fragments obtained from 2-nitro-5-thiocyanobenzoic acid cleavage. In addition to providing a provisional structural map of protein 2.1, we have identified two functional domains of protein 2.1, an 83-kilodalton tryptic peptide (T-83) which binds band 3 and a 65-kilodalton tryptic peptide (T-65) which binds spectrin. We have therefore localized the functional domains along our linear map of protein 2.1.

Erythrocyte membrane proteins 2.1, 2.2, 2.3, and 2.6 [referred to by the nomenclature of Steck (1); also termed syndeins] are a family of sequence-related phosphoproteins ranging in molecular mass from 150 to 210 kilodaltons (kDa), which are peripherally associated with the cytoplasmic surface of the plasma membrane (for reviews, see refs. 1 and 2). The progenitor polypeptide protein 2.1 (also termed ankyrin) is converted into the other family members by a cascade of proteolytic events that occur on the cytoplasmic membrane surface (3). The syndeins bind spectrin to the membrane (2) by associating with the β subunit of spectrin tetramers (4) ~200 Å from the junctional ends of the heterodimers (5). The stoichiometry in situ is one syndein bound per spectrin (αβ2) tetramer (6). The syndeins in turn are associated with the NH2-terminal cytoplasmic domain of the integral membrane glycoprotein band 3 (7, 8). Therefore, the syndeins function in linking the membrane skeleton to the bilayer by linking spectrin to band 3.

Protein 2.1 can be isolated free of proteins 2.2 to 2.6 and other erythrocyte membrane proteins by high-salt extraction of spectrin-depleted inverted vesicles, followed by ion-exchange chromatography (5). In this report, we describe the identification of chemical domains within protein 2.1 defined by limited proteolytic digestion at 0°C. The linear alignment of these fragments along the protein 2.1 molecule has been determined by high resolution two-dimensional peptide mapping of overlapping peptides obtained by proteolytic or chemical cleavage. We have also identified the domains of protein 2.1 involved in binding band 3 and spectrin.

MATERIALS AND METHODS

Purification of Protein 2.1. Purification of protein 2.1 from human erythrocyte membranes was carried out as described (8). The purified protein was stored frozen at ~20°C in 5 mM sodium phosphate/0.2 M KCl/1 mM EDTA, pH 7.6.

Gel Electrophoresis. One-dimensional NaDodSO4/PAGE was performed in 10% polyacrylamide slab gels by the discontinuous system of Laemmli (9). Gels containing linear gradients of acrylamide had gradients ranging from 10% to 15%. Two-dimensional PAGE was carried out according to a modification of the O’Farrell method (10). Isoelectric focusing (IEF) gels (100 mm in length) were prepared in glass tubes [125 × 5.0 mm (inside diameter)] as follows. Nine grams of ultrapure urea (Schwarz/Mann) and 0.15 grams of Triton X-100 (Sigma) were dissolved at 37°C in 29.1% acrylamide/0.9% methylenebisacrylamide, 2.5 ml each, diluted with H2O to a total volume of 14 ml. Amberlite MB-3 (2 g; Sigma) was added to the solution, which successively was stirred at room temperature for 15 min. The ion exchanger was removed by filtration on a MILLEX-HA 0.45-μm filter (Millipore). Ampholines (250 μl each; Pharmacia) covering the pH range pH 5–7, pH 3.5–10, and pH 4–6, and 225 μl of 0.004% riboflavin (Sigma) were added to the filtered solution. Gel solutions were overlayed with 100 μl of 8 M urea and polymerized under light for 4 hr. The top of the gels were rinsed with 100 μl of sample buffer [9.5 M urea/2% (wt/vol) Triton X-100/1% dithiorthioleotol, and 0.8% each of Ampholines of pH 5–7, pH 3.5–10, and pH 4–6]. Lyophilized salt-free samples were dissolved in 100 μl of the sample buffer and loaded on the gels. The samples were overlayed with 100 μl of sample buffer diluted 8:2 with water. Focusing was carried out at room temperature for 16 hr at 350 V with 0.1 M H3PO4 and 0.02 M NaOH as electrode buffers. Gels were removed from the glass tubes with 8 M urea and equilibrated 30 min in the equilibration buffer (3% NaDodSO4/4 mM EDTA/10% glycerol/1% dithiorthioleotol/0.01% bromphenol blue). Gels were cemented to the top of 10–15% gradient polyacrylamide slab gels with 1% agarose (Bio-Rad) dissolved in the equilibration buffer. Electrophoresis in the second dimension was carried out at a constant current of 25 mA per gel. All NaDodSO4/polyacrylamide gels were stained and destained as described by Fairbanks et al. (11).

Analytical IEF. IEF was carried out in Ampholine polyacrylamide gel plates, pH 3.5–9.5 (LKB). Proteins were focused at 10°C for 2 hr at a constant power of 3 W. Focused proteins were fixed and stained according to the LKB IEF instruction manual (LKB). Isoelectric points were determined using an IEF pl calibration kit containing proteins that covered the pl range 2.5–10.5 (Pharmacia).

Abbreviations: IEF, isoelectric focusing; kDa, kilodalton; NTCB, 2-nitro-5-thiocyanobenzoic acid.

*To whom reprint requests should be addressed.
**RESULTS**

Restricted Proteolytic Digestion of Protein 2.1. In order to produce intermediate-size protein fragments, purified protein 2.1 (Fig. 1A, lane b) was subjected to digestion by trypsin and \( \alpha \)-chymotrypsin at 0°C. The time courses for the two proteolytic reactions are shown in Fig. 1. Tryptic digestion (Fig. 1B) resulted in accumulation of two intermediate-size protease-resistant fragments with apparent molecular masses of 83 kDa (T-83) and 65 kDa (T-65), respectively. Also a small fragment that migrated just behind the tracking dye was produced in high yield. By using a 10–20% polyacrylamide gradient gel, the molecular mass of this fragment was calculated to be 11 kDa (data not shown). In addition, several fragments in the molecular mass range of 35–60 kDa were produced in lower yield. Digestion with \( \alpha \)-chymotrypsin resulted in two intermediate-size fragments in high yield (Fig. 1C). The larger fragment had an apparent molecular mass of 83 kDa (C-83), similar to the largest fragment from the tryptic digest. The smaller fragment had an apparent molecular mass of 72 kDa (C-72). Aliquots taken from the chymotryptic digest at 5, 10, 20, and 30 min revealed a large intermediate fragment of 175 kDa (C-175) that was broken down upon further digestion. The 83-kDa region from the tryptic and the \( \alpha \)-chymotryptic digest were composed of many sharp bands representing fragments with minor differences in electrophoretic mobility in the NaDodSO4/polyacrylamide gel. Peptide

![Fig. 1. Enzymatic digestion of protein 2.1. Purified protein 2.1 (A, lane b) was digested at 0°C with trypsin (B) and \( \alpha \)-chymotrypsin (C) in an enzyme-to-substrate ratio of 1:20. At various intervals, aliquots (100 \( \mu \)g of protein) were withdrawn from the incubations and subjected to NaDodSO4/PAGE in 10–15% polyacrylamide gels. In this gel system, uncleaved protein 2.1 remains at the top of the separating gel and, therefore, is not shown. (A) Coomassie Blue-stained erythrocyte ghost proteins (lane a) and purified protein 2.1 (lane b) electrophoresed in a 10% polyacrylamide gel, showing the high degree of purity of the isolated protein 2.1.](image-url)
mapping analysis of individual, stained fragments cut from within the 83-kDa region were identical, showing that the entire band represents a specific region of protein 2.1 (data not shown).

Two-Dimensional PAGE of Protein 2.1 Fragments. Tryptic and α-chymotryptic digestions of protein 2.1 were stopped after 60 min and 30 min, respectively, and subjected to two-dimensional IEF/PAGE (Fig. 2 A and B). In order to trap the C-175 α-chymotryptic fragment in high enough concentration for peptide mapping analysis, the gel was intentionally overloaded with protein from the α-chymotryptic digest. The tryptic fragments T-83, T-65, and T-11 appeared in the second dimension as shown in Fig. 2A. The small T-11 fragment moved just behind the tracking dye and focused with an apparent pI of 4.8 near the acidic end of the urea gel. The T-83 and T-65 protein fragments focused in the urea gel as multiple spots with minor differences in isoelectric points. The T-83 and the T-65 fragments focused in the apparent pI ranges 7.5–8.0 and 6.2–6.8, respectively. Two-dimensional PAGE of the overloaded α-chymotryptic fragments is shown in Fig. 2B. The fragments C-83 and C-72 also focused as arrays of spots in the same apparent pI ranges as observed for the large tryptic fragments. The intermediate fragment C-175 appeared in much smaller concentration than did C-83 and C-72. The NTCB cleavage products of protein 2.1 also were separated by two-dimensional IEF/PAGE (data not shown) for use in peptide mapping studies.

Peptide Mapping Analysis of Protein 2.1 Fragments. The individual Coomassie-stained fragments in an array of spots differing only in isoelectric point were found to have identical peptide maps (data not shown). This applied to all protein 2.1 fragments studied; therefore, the center portion of every array was cut from the gel and used to represent the various fragments. In order to sequentially align the fragments in protein 2.1, peptide maps of T-83, T-65, T-11, C-175, C-83, and C-72 were utilized. In addition, two overlapping fragments that were obtained from NTCB cleavage of protein 2.1 were used. These fragments had molecular masses of 85 kDa (NTCB-85) and 80 kDa (NTCB-80), respectively. The peptide maps of the three major tryptic fragments are shown in Fig. 3. They comprise 76% of the molecular mass of protein 2.1. Peptide maps of T-83 (Fig. 3) and C-83 (not shown) were nearly identical. Thus, at 0°C, trypsin and α-chymotrypsin appear to release similar 83-kDa fragments from protein 2.1. Because of the different substrate specificities of trypsin and α-chymotrypsin, some differences between C-83 and T-83 would be expected; however, the small regions that differ might contain few ioditable tyrosine residues. The peptide map of C-72 (not shown) contained the entire map of T-65 (Fig. 3). Three extra spots were found on the C-72 map, which must represent the extra polypeptide (7 kDa) missing in the T-65 fragment.

Fragments overlapping the tryptic fragments are C-175, NTCB-85, and NTCB-80. Their respective peptide maps and the map of 2.1 are shown in Fig. 4. The map of C-175 contains the entire maps of T-83 and T-65. However, a few additional spots are present on the C-175 map. These spots must come from a 27-kDa polypeptide segment that is not a part of either T-83 or T-65. The map of C-175 presents evidence that T-83 and T-65 are either adjacent or maximally separated by a region of 27 kDa. The map of NTCB-80 shows that T-83 and T-65 are probably not adjacent because 60% of the NTCB-80 map is found on the T-83 map and only 30% of the T-65 map. The remaining 10% of the spots must be derived from the polypeptide region separating T-83 and T-65, or represent the new terminal ends of NTCB-80. Three of these additional spots on the NTCB-80 map are found among the spots on the C-175 map that could not be seen on the T-83 and T-65 maps. The C-175 map also contains three other extra spots (shown with arrows in Fig. 4) not found in the T-83, T-65, or NTCB-80 maps, which however correspond to the three extra spots on the C-72 map when this map is compared to the T-65 map.

It is concluded that T-83 and T-65 are separated by a polypeptide piece of molecular mass < 27 kDa and that C-72 extends closer to a chemical end of protein 2.1 than does T-65. C-175 does not contain any of the limit fragments found on the map of the acidic T-11 fragment. Therefore, T-11 must be within 35 kDa of an end of the protein. The map of NTCB-85 had no spots in common with the T-65 fragment, but 23% of the spots are found on the map of T-83. Thus, the NTCB-85 fragment overlaps the T-83 fragment and, in addition, contains the remaining polypeptide piece that together with the C-175 fragment constitutes the entire protein 2.1 molecule. The entire map of the acidic fragment T-11 is found in the NTCB-85 map. This is consistent with the observation that NTCB-85 in two-dimensional PAGE focused as an acidic fragment in the apparent pH range 5.4–5.6 and places T-11 on the opposite end of the molecule from T-65 (or C-72). Therefore, the consecutive order of the tryptic fragments is T-11, T-83, and T-65. T-11 and T-83 are separated by a protease-sensitive region that can be found in the NTCB-85 fragment, and T-83 and T-65 are separated by another protease-sensitive region that can be found in the NTCB-80 fragment.
Purification of Tryptic Fragments. Gel filtration on Sepha-
cryl S-200 of a trypsin digest of purified protein 2.1 (6.3 mg) resulted in three protein peaks (Fig. 5). The elution profile is consistent with the electrophoretic picture of the digest seen after NaDodSO₄/PAGE, which revealed three fragments in high yield (Fig. 1A). NaDodSO₄/PAGE of protein from peaks A and B confirmed that the fragments T-83 and T-65 were present in these peaks, respectively (Fig. 5 Inset). The recovery of T-83 and T-65 from this gel filtration column was 90% and 75%, respectively. Peak C contained the acidic fragment T-11, which migrated just behind the tracking dye on NaDodSO₄/PAGE (10% polyacrylamide) (Fig. 5 Inset). By utilizing the acidic properties of the T-11 fragment, the fragment could be purified free from other small degradation products by simply eluting the fragment through a Whatman CM 52 cation-exchange column in 0.1 M sodium acetate (pH 5.3). The fragment was recovered in 88% yield. The purity

Fig. 4. (Lower) Peptide mapping of overlap fragments. Overlap fragments of the ¹²⁵I-labeled fragments C-175 (Fig. 2B), NTCB-85, and NTCB-80 were used to align the tryptic fragments in the protein 2.1 sequence. NTCB-85 and NTCB-80 were obtained by NTCB cleavage of protein 2.1. Also shown is the tryptic map of ¹²⁵I-labeled protein 2.1. (Upper) Tracings of the spots observed on the overlap fragments. The tracings have been coded to indicate which spots on the tryptic maps are found on the overlap maps: ○, T-11; ●, T-83; □, T-65. The arrows in C-175 represent those tryptic peptides that are not found in T-83, T-65, or NTCB-80 but are found in the map of C-72.

Fig. 5. Gel filtration of trypic fragments. Purified protein 2.1 (6.3 mg) was digested 60 min at 0°C with trypsin and subjected to gel filtration on Sephacyrl S-200. Inhibition by trypic fragments in the various column fractions of protein 2.1 binding to band 3 or spectrin was measured as described. Inhibition of the protein 2.1–band 3 interaction (○) and of protein 2.1–spectrin binding (△) by the various column fractions is presented as well as their absorbancy at 280 nm (●). (Inset) NaDodSO₄/PAGE in 10% gels of isolated fragments in peak A, B, and C. Vₒ indicates void volume, which was determined with blue-dextran. Maximal binding for the control samples in the two assays were 64 μg of spectrin bound per mg of inverted vesicle protein and 35 μg of protein 2.1 bound per mg of stripped vesicle protein.

Fig. 6. IEF of trypic fragment T-11. Analytical IEF was carried out under nondenaturating conditions. The pH gradient was determined by using standard proteins with known isoelectric points (lane A). Lane B contains 12 μg of purified fragment T-11, which has an isoelectric point of 4.35.
was tested by IEF under non-denaturing conditions (Fig. 6). The fragment focused on an isoelectric point of 4.35. Also two faint bands on each side of the T-11 band were seen. The presence of these bands may reflect that small differences in isoelectric properties exist in this fragment, as has been observed with the larger fragments studied.

Inhibition by Tryptic Fragments of Protein 2.1 Binding to Spectrin and Band 3. The fragments T-83, T-65, and T-11 were tested for their abilities to inhibit protein 2.1 binding to band 3 and spectrin. Aliquots from the Sephacyr S-200 column fractions were used in the spectrin/spectrin-depleted inverted vesicle (protein 2.1) assay and the protein 2.1/syn-dein-depleted inverted vesicle (band 3) assay system. The results are presented as inhibition profiles in the elution diagram shown in Fig. 5. The T-83 fragment clearly inhibited binding of protein 2.1 to band 3. The most concentrated fraction from peak A resulted in 94% inhibition. The T-83 fragment did not inhibit protein 2.1 binding to spectrin. This binding was found to be inhibited by the T-65 fragment. The greatest inhibition measured in fractions from peak B was 40%. The acidic fragment T-11 had no effect in either of the two assay systems. Thus, the binding sites for band 3 and spectrin in protein 2.1 are localized in the tryptic fragments T-83 and T-65, respectively.

DISCUSSION

Three intermediate-size tryptic peptides (T-83, T-65, and T-11) and two chymotryptic peptides (C-83 and C-72) of protein 2.1 are relatively resistant to further proteolytic digestion under our conditions. These chemical domains have been aligned into the structural model presented in Fig. 7, based upon our peptide mapping analysis of each domain and several larger overlapping fragments (C-175, NTCB-80, and NTCB-85). The acidic T-11 fragment must be within 35 kDa of one end, as it is not a portion of the C-175 overlapping fragment. The T-65 fragment must be toward the other end of the molecule, as it does not overlap the NTCB-85 acidic fragment, which contains the entire T-11 fragment—a unique protease-sensitive region not found in the tryptic fragments—and a portion of the T-83 fragment. The NTCB-80 fragment overlaps a portion of both the T-83 and T-65 fragments in addition to a protease-sensitive region that separates these tryptic peptides. All of the limit peptides found on the high-resolution peptide map of protein 2.1 can be accounted for on the maps of the fragments presented in Fig. 7.

In addition to providing a preliminary structural map of protein 2.1, two functionally unique domains of protein 2.1 have been defined. We have demonstrated that T-83 and T-65 contain complete or partial binding sites for band 3 and spectrin, respectively. It is interesting that T-65 and C-72 share nearly identical peptide maps because several years ago a 72-kDa chymotryptic fragment released from spectrin-depleted inverted vesicles was demonstrated to contain a high-affinity spectrin binding site (14), and this 72-kDa fragment was used to identify the syndeins or ankryins as the spectrin-binding proteins (15, 16). Apparently this historically important 72-kDa fragment and C-72 are identical spectrin-binding domains of protein 2.1.

The authors acknowledge the assistance of Jeanette Schwartz in preparing the manuscript and David Stiler for photography. This work was supported by National Institutes of Health Grants HL26059 and NS19357 to S.R.G., who is an Established Investigator of the American Heart Association.