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Tissue-Specific Analogues of Erythrocyte Protein 4.1 Retain Functional Domains

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Analogues of the human erythroid membrane skeletal component protein 4.1 have been identified in perfused rat tissues and human T and B lymphocyte cell lines. olyclonal antibodies were used which are specific for all domains of protein 4.1, the spectrin-actin-promoting 8-Kd peptide, the membrane-binding 30-Kd domain, and the 50-Kd domain. Antibody reactivity, by Western blotting of tissue homogenates, shows reactivity with proteins varying in molecular weight from 175 Kd to 30 Kd. Further, these protein 4.1 analogues appear to be expressed in a tissue-specific fashion. Of the analogues detected there appear to be at least three classes: analogues containing all erythroid protein 4.1 domains, analogues containing all domains but with modified antigenic epitopes, and analogues containing only some domains. Chemical cleavage at cysteine linkages indicates that in analogues containing the 30-Kd region the location of cysteine is highly conserved. This datum suggests that in nonerythroid 4.1 isoforms of higher molecular weight the additional protein mass is added to the amino terminal end (30 Kd end).

Key words: membrane skeleton, nonerythroid protein 4.1 homologues immunoreative isoforms

In erythrocytes, spectrin forms an interconnecting meshwork by self-association to form oligomers [1] and multivalent cross-linking of actin [2]. The spectrin-actin skeleton further associates with and topographically fixes a number of transmembrane proteins [3,4]. The association of the membrane skeleton with transmembrane receptors is mediated by two proteins. Ankyrin or band 2.1 binds close to spectrin's oligomerization site and then further associates with band 3 [4], linking the complex to the membrane. The opposite end of spectrin contains the actin binding site [5]. Spectrin and actin associate only weakly by themselves and protein 4.1 is needed for high-affinity binding [6–8]. Protein 4.1 not only mediates spectrin-actin associations but also links the resulting complex to the membrane through an association with glycophorin [9,10]. The association between glycophorin and protein 4.1 is tightly regulated by the metabolism of a specific phospholipid, phosphatidylinositol-4,5-diphosphate (PIP_2). This phospholipid is an obligate cofactor which binds to glycophorin and forms a high-affinity binding site for

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protein 4.1 on the membrane [11]. Protein 4.1 is also phosphorylated by protein kinase C, Ca^{++}-activated protein kinase, cAMP-stimulated kinase, and a membrane-bound kinase [12–14], possibly resulting in regulation of function [15]. In the erythrocyte, protein 4.1 is crucial in assembly of the membrane skeleton and appears to be the focus of many regulatory mechanisms which may affect cytoskeletal assembly and cell shape [16,17].

The existence of nonerythroid forms of spectrin [18–20], ankyrin, and band 3 [reviewed in ref. 4] have been shown in many cell types, suggesting that diverse cells have common membrane skeletal components. The nonerythroid spectrins are not solely involved in stabilizing the bilayer, as is red cell spectrin. Instead nonerythroid spectrins appear to be more involved in orientation, clustering, and distribution of membrane receptors [21–24], possibly with the involvement of intermediate filaments [25]. This raises the question: Why do spectrin analogues which are similar have such a diversity of function? The answer may be intrinsic to the multiple β and α subunits of nonerythroid spectrins [28–30]. However, more important may be the contribution made by mediators of spectrin function, which are ankyrin and protein 4.1, or their analogues.

Ankyrin analogues have been identified in a number of cell and tissue types, including mammalian and avian [4]. Protein 4.1 has also been found in nonerythroid tissues [31,32, reviewed in 3,4]. Of the tissues or cells in which protein 4.1 has been identified, only the avian erythrocyte and the avian or mammalian lens have demonstrated isoforms of molecular weight greater than 80 Kd [32,33]. The nonerythroid forms of 4.1, like nonerythroid spectrin, show a distribution that is not restricted to the plasma membrane. Indeed, there is evidence that 4.1 analogues associate with stress fibers, tubulin, nuclear membranes, and synaptic vesicles as well as the plasma membrane [34–37]. However, a systematic study of the distribution of protein 4.1 in mammalian tissues or detection of functional domains within protein 4.1 analogues has not been undertaken.

In this study, we have surveyed a variety of tissues as well as T and B lymphocytes, and in each we have identified analogues of erythrocyte protein 4.1. As with avian erythrocytes [33], each tissue or cell type was found to contain multiple variants differing in molecular weight or immunoreactivity. Unlike avian analogues, mammalian nonerythroid protein 4.1 appears to have wisespread occurrence in tissues and may be ubiquitous to all cells. Further, the expression of 4.1 analogues is very tissue specific, both in molecular-weight- and domain-specific immunoreactivity.

MATERIALS AND METHODS

Polyacrylamide Gel Electrophoresis

One-dimensional SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [38] essentially as described previously [39]. Separating gels were of two types—either straight 10% acrylamide with 0.27% N,N′-methylene-bisacrylamide or gradients of 7–15% acrylamide. SDS sample buffer is 3% SDS, 2.25 M urea, 60 mM Tris, 2 mM Na_2EDTA, 100 mM β-mercaptoethanol (β-ME), and 0.004% bromophenol blue. The molecular weight standards were human red cell membrane proteins and in addition for immunoblots proteolytic digests of protein 4.1 and the chicken erythrocyte protein 4.1 isoforms [33].

Antibody Production

Human erythrocyte protein 4.1 was isolated by the method of Tyler et al. [5]. For immunization, protein 4.1 was further purified by preparative SDS-PAGE; the protein 4.1 band was visualized by incubating the SDS-polyacrylamide gel in 1 M KCl. The protein 4.1 band was then excised and electroeluted from the gel [40]. The protein was extensively dialyzed against phosphate-buffered saline (PBS) and then mixed with Freund's complete adjuvant 1:1 to a final concentration of 0.5 mg/ml. Female New Zealand white rabbits were bled for preimmune sera and then injected subcutaneously (30 sites) and intramuscularly (four sites) with 300 μg of protein 4.1. Intramuscular booster injections were given at days 30 and 44; they also consisted of 300 μg, but in Freund's incomplete adjuvant. Blood was collected 2 wk after the second boost. The serum was complement depleted and made 5 mM in NaN_3. In some cases, the IgG was fractionated by precipitation with ammonium sulfate at 40% saturation. The 30-Kd membrane binding domain of protein 4.1 was prepared from pure protein 4.1 by proteolysis with α-chymotrypsin at an enzyme to substrate ratio of 1:20 and a protein 4.1 concentration of 1 mg/ml in 10 mM Tris, 1 mM EDTA pH 8.0. The mixture was incubated for 30 min at 0°C. Under these conditions the 30-Kd domain is resistant to proteolysis, remaining intact as the highest molecular weight peptide and facilitating purification by HPLC gel filtration in 8 M urea, 0.1 M KCl, 10 mM Tris, and 1 mM EDTA, pH 8.0. Antibodies toward the 30-Kd membrane-binding domain were prepared as above. Antibodies specific for the 8-Kd spectrin-actin-promoting peptide were prepared by synthesizing two peptides of 15 and 13 amino acids corresponding to amino acid residues 1–15 (peptide A) and 46–59 (peptide B) from the 8-Kd sequence [40]. During synthesis, an additional cysteine residue was added to the COOH-terminal ends and these were used to independently link the peptides to keyhole limpet hemocyanine (KLH) by using the bifunctional reagent sulfo-MBS (m-maleimidobenzoylsulfosuccinimide ester) in 50 mM phosphate buffer, 1 mM EDTA, pH 7.0. Rabbits were immunized as above with 1 mg of KLH-peptide and boosted with 0.5 mg KLH-peptide. All antibodies were affinity purified on columns containing protein 4.1 or the 30-Kd domain of protein 4.1 coupled to Sepharose CL-4B by cyanogen bromide. After binding the antibody to the column, the column was washed with 1 M KCl, 30 mM Tris, pH 7.6, to elute nonspecifically bound antibody. Specifically bound antibody was then eluted with 1 N acetic acid, 0.15 M NaCl, pH 4.0, and dialyzed into PBS. Antibodies from each rabbit were purified on a column which remained committed to that antiserum. Antibodies specific for the 50-Kd domain were prepared by passing the anti-4.1 antibody through a column containing the 30-Kd domain coupled to Sepharose; the antibody was cycled until no further anti-30-Kd antibody could be detected in the flow-through.

Preparation of Tissues

Tissues were prepared from adult Sprague-Dawley rats which were anesthetized with ether and extensively depleted of red cells by vascular perfusion through the left chamber of the heart. The perfusion buffer was phosphate-buffered saline (PBS) (0°C) containing 5 mM EGTA, 1.0 mM diisopropylfluorophosphate (DFP), 0.3 mM N-p-tosyl-lysine methyl ester (TLCK), 2 μg/ml leupeptin, 15 μg/ml benzamidine, 2 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.35. The protease inhibitors were added to minimize proteolysis of the protein 4.1 analogues, which in

some tissues are very labile. Leupeptin was found to be particularly important for the inhibition of proteolysis of the brain analogues.

After perfusion the tissues were surgically removed and placed on ice until homogenization. Then 2 volumes of 9 M urea, 12% SDS, 250 mM Tris, 8 mM EDTA, 1 M β-ME, pH 6.9, was added to the tissue sample and homogenized with a glass-on-glass Dounce homogenizer in a boiling water bath. Homogenization of whole tissues in this fashion appeared to shear the DNA, making the samples more manageable, but did not alter the pattern of immunoreactive species (see chicken erythrocyte protein 4.1). Estimates of protein content were made by dialyzing a known volume of tissue homogenate against a 0.1% SDS solution; protein was then determined by the method of Lowry et al. [50]. Cleavage of protein 4.1 by α-chymotrypsin and 2-nitro-5-thiocyanobenzoic acid (NTCB) was according to Leto and Marchesi [47]. NTCB cleavage of tissues was accomplished by dialyzing solubilized tissues against 10 mM Tris, 1 mM EDTA, 0.1% SDS, pH 8.0. The protein concentration of the sample was analyzed by Lowry's method [50]. The sample was then lyophilized and resolubilized in 7.5 M guanidine, 50 mM Tris, 1 mM EDTA, and 2 mg/ml NTCB, incubated 30 min at 20°C, titrated to pH 9.0 with 1 M Tris, and incubated at 37°C for 12 hr. The reaction was stopped with 20 mM β-ME, and the solution was dialyzed against 0.1% SDS and 1 mM EDTA and then diluted with sample buffer for SDS-PAGE.

Fractionation of Parotid and Brain Protein 4.1 Analogues

The brain and the parotid were both isolated from perfused rats as above. The brain was freed of the meninges and white matter and then homogenized in 320 mM sucrose (1 g/10 ml), 4 mM HEPES, 5 mM EGTA, 3 μg/ml leupeptin, 0.5 mM PMSF, 0.2 mM DFP, pH 7.0 on ice [42,43]. A rotating Teflon-on-glass Potter-Elvehjem tissue grinder was used to break up tissue. The homogenate was centrifuged at 900g for 5 min to pellet cells and nuclei; the postnuclear supernate was collected and centrifuged at 30,000g for 30 min. The resulting pellet was washed once in homogenization buffer and resuspended in 40 mM KCl, 10 mM sodium phosphate, 2.0 mM EGTA, 2 mM β-ME, 1 mM NaN_3, 2 μg/ml leupeptin, and 0.5 mM DFP, pH 7.4. The crude membrane pellet was homogenized by using a glass-on-glass tight-fitting Dounce homogenizer. The membranes were washed twice (washed membranes) at 60,000g for 30 min and then extracted with 1 M KCl in the above buffer for 60 min on ice. The membranes were pelleted at 150,000g for 1 hr (1 M KCl-extracted membranes). The 1 M KCl supernate was dialyzed against the above phosphate buffer without KCl. The washed membranes, 1 M KCl-extracted membranes, and 1 M KCl supernatant were applied to SDS-PAGE and analyzed by immunoblotting for protein 4.1 analogues.

The parotids, in minimal essential medium (MEM), were freed of adipose tissue and homogenized as described earlier [44] in 300 mM sucrose, 2 mM MOPS, 0.2 mM $MgCl_2$, 2 μg/ml leupeptin, 0.1 mM DFP, 0.4 mM PMSF, and 0.2 μg/ml N,N′-diphenyl-p-phenylenediamine (DPPD), pH 7.2, and centrifuged as above to separate nuclear supernate and pellet. The nuclear pellet was rehomogenized and sedimented, and the supernatants were combined. A crude membrane pellet was obtained by centrifuging the nuclear supernate at 140,000g for 120 min; a crude microsomal supernate and pellet were prepared by centrifuging at 50,000g for 90 min. The microsomal supernate was further fractionated to isolate parotid granules [44].

Immunoblotting

Proteins were transferred from SDS-polyacrylamide slab gels to nitrocellulose paper for detection by antibodies [45,46]. After electrophoresis, the SDS-polyacrylamide gel was pressed semirigid against a nitrocellulose sheet (Schleicher and Schuell BA85, 0.45 μm) and transferred at 7 V/cm for 12 hr, in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at pH 8.3. After transfer the sheets were washed in 150 mM NaCl, 50 mM Tris, and 5 mM NaN_3, pH 7.4 (Tris-saline), and blocked in 5% bovine serum albumin (BSA) in the same buffer. Affinity-purified antibody (20 μg/ml) in Tris-saline with 5% BSA was incubated for 2.5 hr at room temperature. The nitrocellulose sheet was then washed in Tris-saline—at least four changes of 100 ml each over 2 hr. Protein A labelled with ^{125}I (1×10^9 cpm/mg) at a concentration of 1×10^6 cpm/ml in 5% BSA and Tris-saline was incubated for 1 hr at room temperature. The sheets were washed with six to eight changes of 100 ml of Tris-saline over 6–12 hr, dried between filter paper, and autoradiographed with Kodak XAR-5 film. For immunoblotting with anti-8-Kd antibody, antibodies against peptides A and B were combined. This gave higher sensitivity and greater cross-reactivity toward protein 4.1 analogues.

RESULTS

Protein 4.1 Antibodies

Previous studies demonstrated that protein 4.1 can be cleaved by proteases into functional and structural domains [39,47]. Four major domains have been characterized, including the 30-Kd N-terminal domain containing the binding site for glycophorin-PIP_2 [3]; a 16-Kd domain, which is phosphorylated [13]; an 8-Kd peptide containing the spectrin-actin-promoting activity [39,41]; and a heterogeneous peptide of 22K and 24K, which is the carboxyl terminus. The 50-Kd domain contains the 16Kd, 8Kd, and the 22–24Kd peptides and runs as a 50-Kd molecular weight peptide. The approach we have taken in studying nonerythroid analogues is to raise polyclonal antibodies to specific functional domains of protein 4.1. With these antibodies, functional domains within protein 4.1 analogues can be detected. In addition, other analogues of protein 4.1 retaining only some of the functional domains can now be selectively detected and studied.

Five sets of antibodies, each with unique specificity, were used to identify protein 4.1 analogues. These antibodies are specific for all domains of protein 4.1 (anti-4.1), the 30-Kd domain (anti-30 Kd), all domains of 4.1 except the 30-Kd domain (anti-50 Kd) and two peptides within the 8-Kd peptide (anti-8 Kd). Anti-4.1 was prepared by using purified protein 4.1 [5] that was further resolved by SDS-PAGE, separated from the polyacrylamide gel by electroelution [40], and used to immunize rabbits. The 30-Kd domain was prepared by α-chymotryptic digestion of isolated protein 4.1 and further purified by HPLC or by SDS-PAGE and electroelution. After purification, both immunogens were single bands on SDS-PAGE at loads of 20 μg per lane. Antibodies toward the spectrin-actin-promoting region of protein 4.1 were prepared by synthesizing peptides which are 15 and 13 amino acids long, from the known sequence of the 8-Kd peptide [41].

The specificity of the antibodies toward domains was determined by proteolytic cleavage with α-chymotrypsin in an increasing ratio of α-chymotrypsin to protein 4.1 [47]. This results in a cascade of cleavages, producing resistant domains or small peptides. Protein 4.1 was also cleaved by the cysteine-specific reagant NTCB; this cleavage is

restricted to the 30-Kd domain containing all of the cysteine residues [49]. Peptides were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with affinity-purified antibodies. The immunoblots (Fig. 1a–d) demonstrate excellent specificity of antibodies toward only protein 4.1 and specific domains toward which they were prepared. The model shown in Figure 2 summarizes the immunoreactivity of the antibodies toward protein 4.1.

Occurrence of Nonerythroid Protein 4.1 in Tissues

Rats were whole body perfused through the left ventricle with severing of the aorta. Particular care was taken to inhibit proteolysis during perfusion by addition of high concentrations of protease inhibitors to the perfusion buffer. Since the nonerythroid analogues of protein 4.1 are labile to protease activity, failure to include protease inhibitors

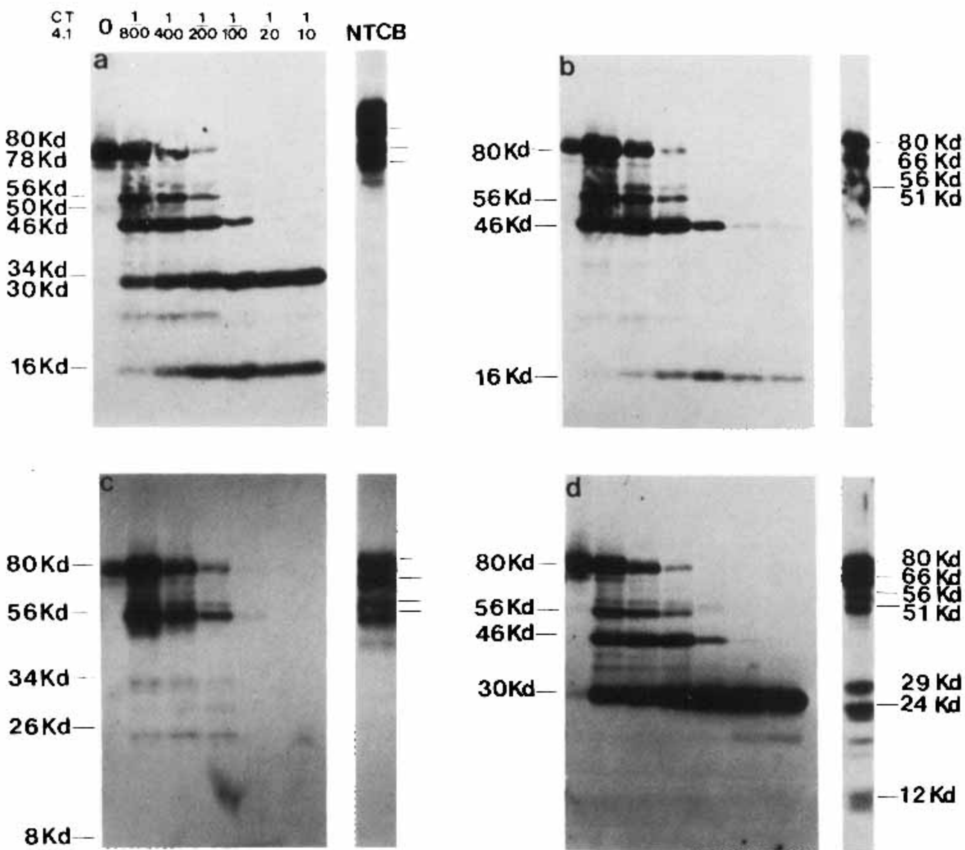

Fig. 1. Domain specificity of the affinity-purified antibodies. The immunoreactivity of antibodies against erythrocyte membranes (first lane); α-chymotryptic digests of protein 4.1, increasing protease concentrations; and NTCB-cleaved protein 4.1. **a:** Polyclonal antibody toward the entire molecule. **b:** Polyclonal antibody specific for the 50-Kd domain lacks anti-30-Kd reactivity. **c:** Polyclonal antibody specific for the spectrin-actin-promoting region of protein 4.1 reacts only with peptides containing the 8-Kd region. **d:** Polyclonal antibody specific for the 30-Kd membrane-binding domain of protein 4.1 reacts only with peptides containing the 30-Kd region. NTCB cleavage indicates that the major antigenic reactivity is within the first 20-Kd region in the 30-Kd domain.

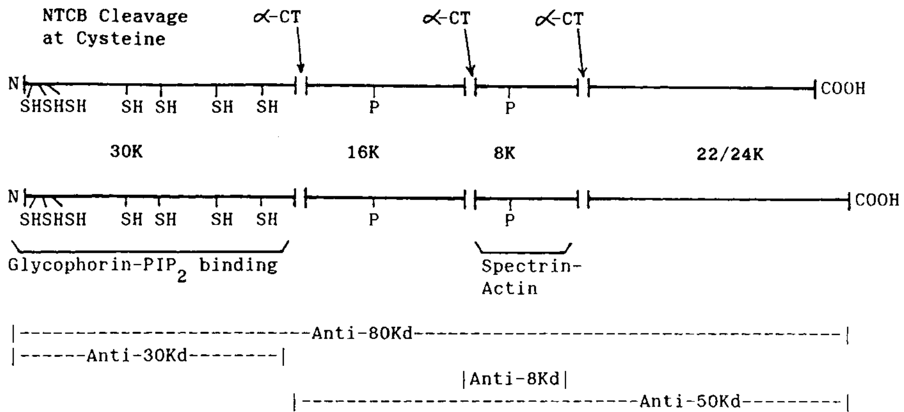

Fig. 2. Structural and functional model of erythrocyte protein 4.1 showing antibody reactivities.

resulted in the loss of immunoreactive forms upon homogenization of some of the tissues (see Fig. 7). Tissues were perfused until the perfusate was clear and free of red cells, after which tissues were surgically removed and rapidly homogenized in hot buffer containing urea, β-mercaptoethanol, and SDS. Protein concentrations were diluted to 5–15 mg/ml, and typically 150 μg of protein was resolved by SDS-PAGE. Analogues of erythroid protein 4.1 were identified by immunoblotting and immunoautoradiography.

Initial experiments with solubilized chicken erythrocytes demonstrated that six immunoreactive proteins of molecular weight 90 Kd, 100 Kd, 120 Kd, 150 Kd, 160 Kd, and 175 Kd could be detected by antiprotein 4.1 antibodies, confirming previous results [33] to the effect that avian erythrocytes contain at least six variants of protein 4.1. Further, the avian variants contained all of the domains found in human erythrocyte protein 4.1, indicating that the chicken protein 4.1 analogues are closely homologous to human erythroid protein 4.1 (Fig. 3). Since the human protein 4.1 antibodies react with all of the chicken protein 4.1 analogues, the chicken variants were used as molecular weight and immunological standards, along with known amounts of human protein 4.1 and proteolytic digests of protein 4.1.

As shown in Figure 4, all of the tissues analyzed contain nonerythroid protein 4.1 analogues. These data are summarized in Table I. Many of the variants differed substantially in molecular weight from that of the 80-Kd erythrocyte protein. The tissues also vary in the content of protein 4.1; skeletal muscle, cardiac muscle, and intestine contain relatively low quantities while kidney, parotid, and pancreas contain relatively large amounts of protein 4.1 analogues. The spleen is also rich in protein 4.1 (80 Kd), but this is likely to be mainly red cell protein 4.1 since the spleen is a difficult organ to perfuse.

A striking observation is that protein 4.1 analogues are expressed in a tissue-specific fashion—for example, in skeletal muscle and cardiac muscle the predominant protein 4.1 analogue (80 Kd), which cross-reacts with antibodies to all domains. Immunoblots of solubilized jejunum show four immunoreactive analogues of molecular weight 80 Kd, 130 Kd, 150 Kd, and 175 Kd, whereas the kidney cortex contains 80-Kd and 130-Kd isoforms. The tissue-specific expression of analogues was observed in all tissues and cell types studied.

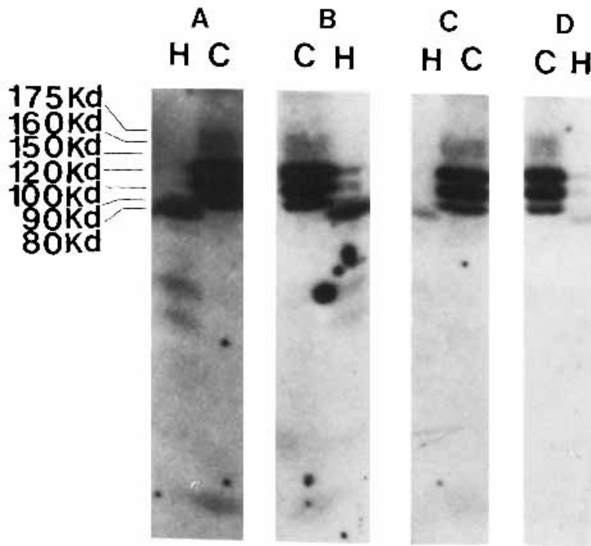

Fig. 3. Immunoreactivity of antibodies toward human erythrocyte protein 4.1 (H) and the chicken protein 4.1 (C) analogues. **A:** Anti-8-Kd antibody. **B:** Anti-50-Kd antibody. **C:** Anti-80-Kd antibody. **D:** Anti-30-Kd antibody. All antiprotein 4.1 antibodies showed the same immunoreactivity toward the chicken analogues. Lanes C and D have 1 ng of erythroid protein 4.1; lanes A and B have 10 ng of erythroid protein 4.1. Each lane contains 100 μg of total chicken erythrocyte protein.

The brain, specifically gray matter, contains the most diverse nonerythroid protein 4.1 isoforms. Immunoblotting brain homogenates using antibodies prepared from multiple rabbits demonstrated that each antibody has a preferential affinity for selected epitopes. In summation, these results indicate that there are at least four immunoreactive analogues in the brain with molecular weights of 115 Kd, 135 Kd, 150 Kd, and 175-Kd, each showing cross-reactivity with at least two antibodies specific for protein 4.1 or domains of protein 4.1. Unlike the immunoreactive species in the other tissues, the brain protein 4.1 analogues, because they appear to contain different immunoreactive epitopes, may be unique species, arising from different genes or by posttranslational processing. Although this is most obvious in the brain, analogs in other tissues appear to have this property—for example, the 175-Kd isoform observed in the adrenal gland, spleen, lymph node, and lymphocytes. Our antibodies, unlike those of Baines and Bennett [35], do not cross-react with synapsin I. This may be because we have crossed between species in our study, whereas Baines and Bennett consistently used porcine tissues. The fact that they did not see higher molecular weight protein 4.1 analogues in the brain may be due to proteolysis since analogues in the brain are very susceptible to proteolytic degradation (see Fig. 7).

Two protein 4.1 analogues did not fit into the general category of protein 4.1 analogues so far discussed. The first is an immunoreactive band at a molecular weight of 30 Kd found in all nonerythroid tissues with the exception of skeletal and cardiac muscle, brain, and the lens. This 30-Kd species shows very strong reactivity with anti-8-Kd antibodies and also reacts with anti-4.1 R1. The second immunoreactive analogue has a molecular weight of about 175 Kd and is similarly distributed. This isoform is detected by both anti-30-Kd antibodies, although anti-30-Kd R2 antibody has the highest

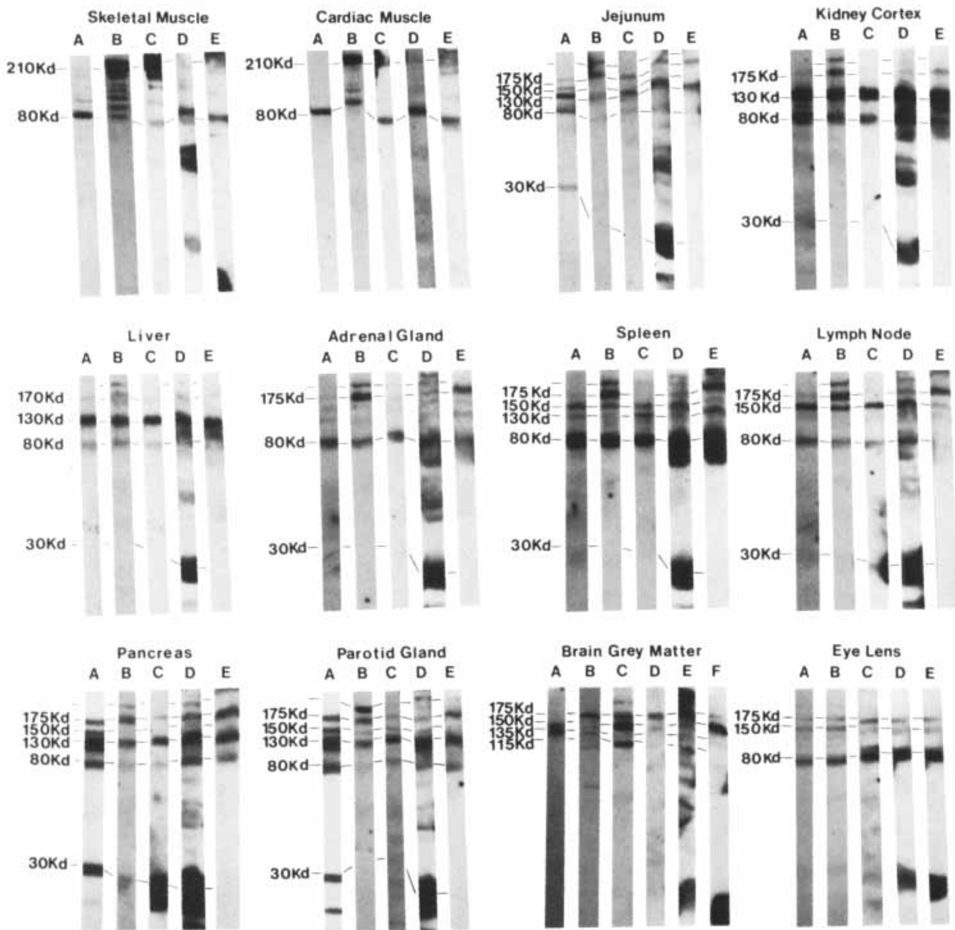

Fig. 4. Detection of protein 4.1 analogues in rat tissue homogenates by polyclonal antibodies specific for protein 4.1 (80 Kd) and domains within protein 4.1. **A:** Polyclonal anti-80-Kd antibody R1. **B:** Polyclonal anti-30-Kd antibody R2. **C:** Polyclonal anti-30-Kd antibody R1. **D:** Polyclonal anti-8-Kd antibody. **E:** Polyclonal anti-50-Kd antibody (from R2). For the brain gray matter antibodies from six rabbits were used. These are: A, Anti-80-Kd R1; B, Anti-80-Kd R2; C, Anti-30-Kd R2; D, Anti-30-Kd R1; E, Anti-8 Kd; F, Anti-50-Kd R2. As discussed in the Materials and Methods, 150 μg of total protein was applied to each lane of the SDS-PAGE and Western blotted.

sensitivity toward this analogue. The anti-4.1 R1 does not detect this analogue. However, the anti-4.1 R2 antibody (not shown) and as the anti-50-Kd antibody show reactivity with this band. The anti-8-Kd antibody also shows reactivity with this isoform, which suggests that indeed it is an analogue of protein 4.1, but with limited antigenic epitopes in common with the erythroid protein. This identical pattern of immunoreactivity is demonstrated more clearly by using the membranes of T and B clonal lymphocytes and comparing these to the spleen and lymph node (Fig. 5). One interpretation of this result is that the 175-Kd analogue is quite different from erythroid protein 4.1 but retains functional protein 4.1 domains.

TABLE I. Immunoreactive Protein 4.1 Analogues in Tissues

Molecular weight (Kd)	Tissue type								
	Cardiac & skeletal muscle	Jejunum	Kidney cortex	Liver	Adrenal gland	Spleen	Lymph node	Pancreas & parotid gland	Brain grey matter	Eye lens
210	30KdR1&R2	30KdR1	30KdR1	30KdR1	30KdR1	30KdR1	30KdR1	30KdR1	—	—
175	—	All domains	All domains selective by 30KdR1	—	All domains selective by 30-Kd R1	All domains selective by 30-Kd R1	All domains selective by 30-Kd R1	All domains	All domains	All domains
170	—	—	—	All domains	—	—	—	—	—	—
150	—	All domains low sensitivity	—	—	—	All domains	All domains	All domains low sensitivity	All domains but all very different sensitivity	All domains
130	—	All domains low sensitivity	All domains	All domains	—	All domains low sensitivity	—	All domains	—	—
135	—	—	—	—	—	—	—	—	All domains low sensitivity	—
115	—	—	—	—	—	—	—	—	30-Kd R1, 8 Kd	—
80	All domains	All domains	All domains	All domains	All domains	All domains	All domains	All domains	—	All domains
30	—	8 Kd	8 Kd	8 Kd	8 Kd	8 Kd	8 Kd	80-Kd R1, 8 Kd	—	—

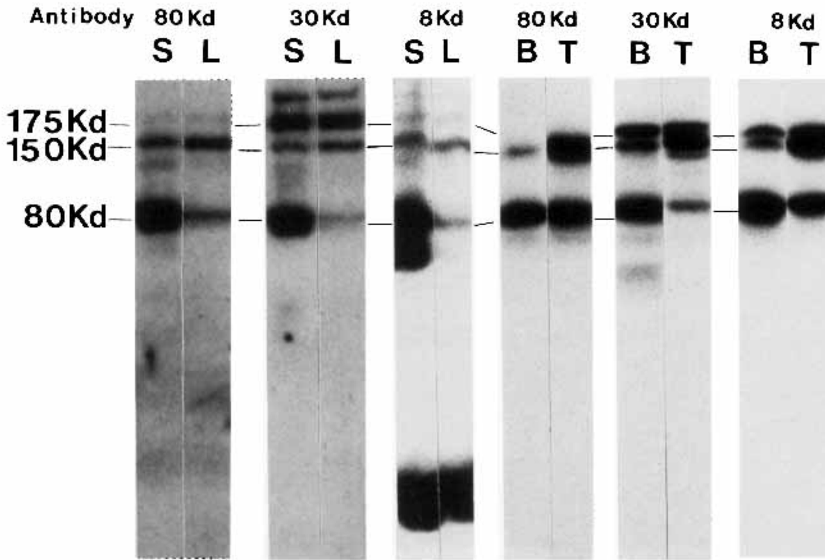

Fig. 5. Immunoblots of spleen (**S**) and lymph node (**L**) homogenates from the rat and **T** and **B** lymphocyte plasma membrane preparations from clonal human cell lines. The blots of spleen and lymph node are of 150 μg of total protein; blots of T and B lymphocyte membranes are of 20 μg of membrane protein.

Fractionation of Parotid Nonerythroid Protein 4.1

The parotid gland was surgically removed from rats perfused with buffer containing protease inhibitors. The tissue was homogenized and cytoplasmic proteins, granules, organelles, and plasma membranes were separated from nuclei and intact cells by differential centrifugation. The nuclear pellet after further washing was homogenized in hot buffer containing urea and SDS. The nuclear supernatant, in 0.3 M sucrose buffer, was centrifuged at 30,000g for 30 min to sediment microsomes [44]. The postmicrosomal supernatant containing granules, plasma membrane, and other organelles was sedimented at 200,000g for 120 min. The resulting pellet and supernatant were homogenized in hot SDS and urea for SDS-PAGE and immunoblotting. The remainder of the postmicrosomal supernate was used to isolate granules to determine if protein 4.1 analogues remain bound to these organelles. Immunoblotting of equivalent amounts of the component fractions using the anti-4.1 antibody and the anti-8-Kd antibody demonstrates that all immunoreactive components fractionate in a similar manner (Fig. 6). The protein 4.1 analogues appear to fractionate equally between the nuclear pellet and the nuclear supernate. Protein 4.1 analogues were not found in the soluble cytoplasmic fractions. Sedimentation of the membranes and organelles in the nuclear supernate demonstrates that all protein 4.1 analogues are bound to membrane or cytoskeletal components which sediment upon centrifugation. Isolated granules did not retain detectable amounts of bound protein 4.1 under these conditions. All of these results are consistent with the immunoreactive species being cytoskeletal or membrane skeletal components. The fact that the 30-Kd component cofractionates with the higher analogues is supportive evidence that this species is a truncated analogue containing the spectrin-actin-promoting region.

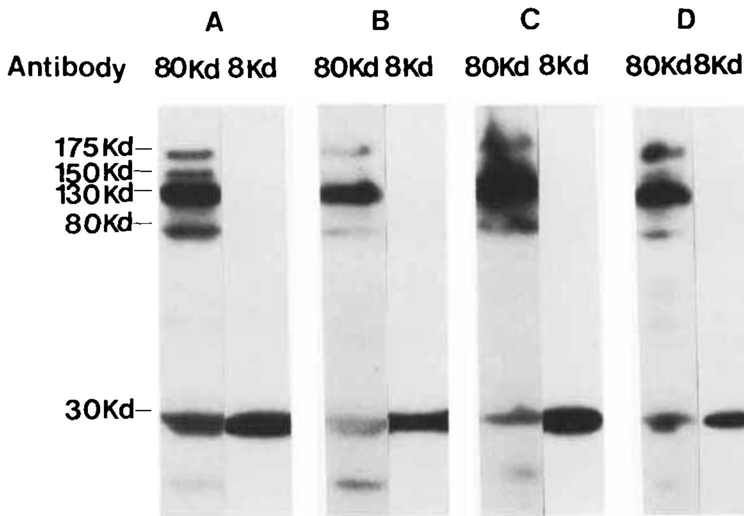

Fig. 6. Fractionation of protein 4.1 analogues in the parotid. **A:** Homogenate, 150 μg protein. **B:** Crude plasma membrane fraction, 50 μg protein. **C:** Nuclear pellet, 50 μg protein. **D:** Total microsomal membrane fraction, 50 μg protein. The protein 4.1 analogues always sedimented with membrane fractions; there were no analogues in the soluble cytoplasmic fraction. Isolated granules did not retain protein 4.1 analogues.

Fractionation of Brain Protein 4.1 Analogues

Brain protein 4.1 analogues were fractionated similar to that of the parotid. To identify protein 4.1 analogues in the brain, it was absolutely required that the brain be perfused with protease inhibitors, specifically leupeptin (DFP was only partly effective), which inhibits Ca^{++}-activated protease [48]. When this step was deleted, only proteolytic fragments of about 60–70 Kd were found in the brain (Fig. 7), demonstrating that isoforms in the brain are very labile to proteolysis. The perfused brain was surgically removed, stripped of the meninges, and homogenized according to De Camilli et al. [43] and Davis and Bennett [42]. A crude membrane fraction was isolated and extracted with high salt by a method analogous to the extraction of protein 4.1 from red cell membranes. Within these conditions, protein 4.1 analogues appear to be membrane or cytoskeletal bound and are to some extent extractable by high salt (Fig. 6). In these studies, the anti-4.1 R2 was used, which has greatest specificity toward the 175-Kd isoform in the brian; the 145-Kd, 120-Kd, and 105-Kd forms are also visualized, although to a lesser extent. The fact that the nonerythroid protein 4.1 analogues, visualized with our antibodies, are so labile to the Ca^{++}-activated protease may be as important functionally as the action of this protease on brain spectrin [48].

Localization of Cysteine in Nonerythroid Protein 4.1

In erythrocyte protein 4.1 the cysteines are completely localized in the 30-Kd N-terminal domain (see Fig. 2). As a result, cleavage with NTCB results in a distinctive pattern of peptides [47]. To determine if the nonerythroid protein 4.1 analogues have retained this localization of cysteines, tissue homogenates were subjected to NTCB cleavage, and the products were resolved by SDS-PAGE and immunoblotted. Anti-4.1

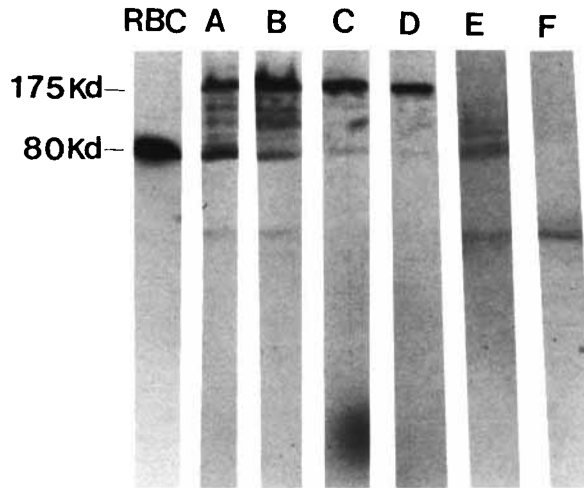

Fig. 7. Fractionation of brain gray matter protein 4.1 analogues. RBC. Rat erythrocyte membranes. **A:** Brain homogenate, 150 A165g protein. **B:** Crude brain membrane fraction, 50 μg protein. **C:** 1 M KCl extract from brain membranes, 10 μg protein. **D:** 1 M KCl-extracted brain membranes, 40 μg protein. **E:** Brain homogenate from rat perfused with buffer containing DFP as the only protease inhibitor, 150 μg protein. **F:** Brain from rat perfused with buffer without protease inhibitor, 150 μg protein. In all cases the brain homogenates were prepared in hot urea and SDS buffer. Polyclonal anti-80-Kd antibody R2 was used for Western blotting.

R1 was used to detect analogues since this antibody appears to detect largely the most erythroidlike higher molecular weight protein 4.1 isoforms. The results (Fig. 8) demonstrate that nonerythroid protein 4.1 analogues in the tissues probed have NTCB cleavage patterns very similar to red cell protein 4.1. These data further support the profile of immunoreactivity with antibodies specific for the 30-Kd domain since they suggest that the 30-Kd region is highly conserved in these analogues. These data also suggest that the additional protein mass present in nonerythroid analogues of higher molecular weight is added to the amino terminal end of the nonerythroid isoforms. This is suggested since all the cysteines in red cell protein 4.1 are located in the 30-Kd domain. The ladder of peptides is a result of incomplete cleavage at cysteines, which is normal for the NTCB reaction. The nonerythroid analogues have very similar cleavage patterns to that of erythrocyte protein 4.1. Most importantly, as with red cell protein 4.1, after NTCB cleavage there are no immunoreactive species of molecular weight greater than 80 Kd. If the additional protein mass of the nonerythroid analogues was added to the N-terminus, this is what would be expected. However, such a result could also occur if the protein mass were added at the carboxyl end if there was a cluster of cysteines early in the carboxyl end sequence.

DISCUSSION

The expression of nonerythroid protein 4.1 analogues in the different tissues studied appears in at least three classes. The first class consists of analogues that are most similar to erythroid protein 4.1, showing reactivity with all antibodies. These forms would be

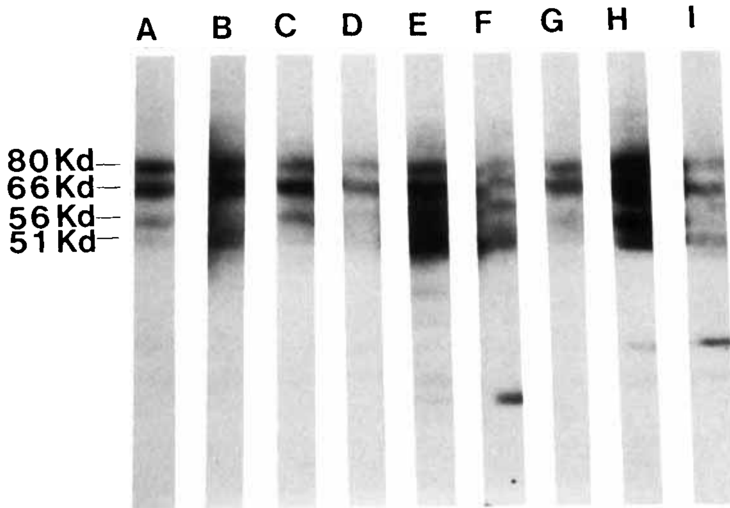

Fig. 8. Immunoreactivity of anti-80-Kd antibody R-1 against NTCB cleavages of tissue homogenates. **A:** Rat erythrocytes. **B:** Skeletal muscle. **C:** Cardiac muscle. **D:** Jejunum. **E:** Kidney cortex. **F:** Liver. **G:** Adrenal gland. **H:** Spleen. **I:** Pancreas. The NTCB digest from 200 μg of protein was loaded on each lane.

analogous to the protein 4.1 isoforms in the avian erythrocyte [33] and the bovine lens [32]. The second class includes isoforms reactive with anti-4.1 antibodies and the domain-specific antibodies, but they have modified epitopes limiting their reactivity to only some antibodies. These would include the 175-Kd isoform found in the intestine, kidney, adrenal, spleen, and lymph node and all of the isoforms found in the brain. The third class of isoforms consists of immunoreactive proteins that show high cross-reactivity with one antibody. These analogues include the 210-Kd form, reactive with anti-30-Kd antibodies, most prominent in skeletal and cardiac muscle. Also in this class is the 30-Kd form that reacts with anti-8-Kd antibody and anti-4.1 R1 and is found in highest quantity in pancreas and parotid.

Those nonerythroid protein 4.1 analogues in the first class are very similar to erythrocyte protein 4.1, and possibly, like the avian protein 4.1 analogues, they are derived from the same gene [31–33]. These analogues appear to contain all of the functional domains found in erythrocyte protein 4.1. The striking similarity between these forms comes from the similarity of peptide maps in comparing erythroid protein 4.1, the avian analogues, and the mammalian analogues in the lens. Further evidence for similarity comes from the NTCB cleavage of nonerythroid protein 4.1 analogues in the tissues. Cysteine-specific cleavage of proteins in the tissues, followed by immunoblotting with anti-4.1 R1, which is most specific for the first class of nonerythroid analogues, suggests that this class of analogues has cysteine residues localized in a fashion almost identical to that of red cell protein 4.1. The fact that analogues of a molecular weight greater than 80 Kd have a NTCB cleavage pattern like the 80-Kd species could be explained by addition of protein at the amino terminal portion of the molecule. In erythrocyte protein 4.1, at the amino terminal end, there are three cysteine residues located within the first 31 amino acid residues; the remaining four cysteine residues are all located within the 30-Kd domain of the 80-Kd sequence [49]. As such,

addition at this termini would lead to NTCB cleavage patterns detected by the anti-4.1 R1 which are similar to the red cell protein.

The retention of similar functional and structural domains suggests that this class of proteins may be functionally related to the erythrocyte protein. Indeed, studies with the lens protein 4.1 analogues [32] imply that these proteins bind to lens spectrin. Immunofluorescence data demonstrate an apparent localization of the lens analogues to the membrane of the epithelial layer [33], which suggests that these proteins, like the red cell protein, may function as mediators of the association of spectrins with actin and the membrane.

All of the tissues tested express an 80-Kd analogue of protein 4.1; however, the concentration of the 80-Kd nonerythroid form varies considerably between tissues. The expression of the 80-Kd isoform appears to be independent of whether or not the tissue is expressing a higher molecular weight isoform. For example, the kidney, which expresses a large amount of 130-Kd analogue, also expresses the 80-Kd isoform in large quantity. In contrast, the liver, which also expresses the 130-Kd protein in comparable amounts, expresses only small amounts of the 80-Kd protein. The pancreas expresses little 80-Kd protein, while producing large quantities of a 130-Kd protein. T and B clonal lymphocyte cell lines both express 150-Kd isoforms, as well as the 80-Kd isoform. The results demonstrate that individual cells have multiple forms of protein 4.1, each independently expressed.

The methods we have used to identify nonerythroid protein 4.1 analogues have very likely focused only on a subset of the total protein 4.1 analogues in nonerythroid tissues. Although the antibodies used here identify a large number of protein 4.1 isoforms differing in molecular weight and immunoreactivity, it is likely that these are forms most homologous to the erythroid protein. In this study protein 4.1 analogues such as synapsin I [35] were not detected by any of the antibodies, which suggests that synapsin I is an example of a molecule which may be related to protein 4.1 but is of lower homology than those identified here. In turn, analogues which stain with all protein 4.1 antibodies but with different intensities may represent isoforms which have retained the major domains of the erythroid proteins but with small insertions or deletions by mRNA splicing mechanisms [51,52]. If this is the case the number of protein 4.1 analogues observed here may be just the tip of the iceberg.

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