Phosphorylation of Ankyrin Down-Regulates Its Cooperative Interaction With Spectrin and Protein 3

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Ankyrin mediates the primary attachment between beta spectrin and protein 3. Ankyrin and spectrin interact in a positively cooperative fashion such that ankyrin binding increases the extent of spectrin tetramer and oligomer formation (Giorgi and Morrow: submitted, 1988). This cooperative interaction is enhanced by the cytoplasmic domain of protein 3, which is prepared as a 45–41-kDa fragment generated by chymotryptic digestion of erythrocyte membranes. Using sensitive isotope-ratio methods and non-denaturing PAGE, we now demonstrate directly (1) the enhanced affinity of ankyrin for spectrin oligomers compared to spectrin dimers; (2) a selective stimulation of the affinity of ankyrin for spectrin oligomer by the 43-kDa cytoplasmic domain of protein 3; and (3) a selective reduction in the affinity of ankyrin for spectrin tetramer and oligomer after its phosphorylation by the erythrocyte cAMP-independent membrane kinase. The phosphorylation of ankyrin does not affect its binding to spectrin dimer. Ankyrin also enhances the rate of interconversion between dimer-tetramer-oligomer by 2–3-fold at 30°C, and in the presence of the 43-kDa fragment, ankyrin stimulates the rate of oligomer interconversions by nearly 40-fold at this temperature. These results demonstrate a long-range cooperative interaction between an integral membrane protein and the peripheral cytoskeleton and indicate that this linkage may be regulated by covalent protein phosphorylation. Such interactions may be of general importance in nonerythroid cells.

Key words: protein phosphorylation, cytoskeleton, erythrocyte

The mammalian erythrocyte cytoskeleton is composed of a peripheral anastomosing array of proteins closely applied to the cytoplasmic face of the plasma membrane. Its principal components include spectrin, actin, and protein 4.1, as well as several less-well-characterized components [for reviews, see 1,2]. The predominant lateral associations within this lattice involve homotropic interactions between spectrin heterodimers at one end of the spectrin molecule and a heterotropic interaction with actin at the other

end. The interaction of spectrin with actin is stimulated by protein 4.1, which appears to act specifically on the beta subunit of spectrin [3]. Recently, it has been demonstrated that ankyrin will stimulate the formation of spectrin tetramers and oligomers [4] as well as bind selectively to spectrin tetramer in preference to spectrin dimer [4–6]. Thus, it appears that both of the dominant lateral associations within the erythrocyte membrane skeleton (spectrin-spectrin and spectrin-actin) are subject to modulation by a third protein (ankyrin or protein 4.1, respectively). Covalent phosphorylation has also been found to down-regulate the interaction of protein 4.1 with spectrin-actin [7,8] and of ankyrin with spectrin tetramer [6].

The membrane skeleton is joined to the lipid bilayer by way of specific interactions with integral membrane proteins. The strongest such "vertical" interaction involves the binding of the cytoplasmic domain of protein 3 to ankyrin, which is then bound to the beta subunit of spectrin [2,9]. Other interactions also exist [reviewed in 1,2,10]. Significantly, the cytoplasmic domain of protein 3 has been recently shown to enhance the affinity of ankyrin for the larger oligomeric forms of spectrin, and correspondingly, to stimulate spectrin oligomer formation in vitro [4]. Thus it appears that a long-range interaction exists in the erythrocyte membrane skeleton between a "vertical" association of ankyrin with protein 3 and a "lateral" association between the spectrin heterodimers. The effects of ankyrin phosphorylation on this interaction are unknown, as are the molecular mechanism by which it operates.

In the present report it is found that ankyrin selectively binds to spectrin tetramer and oligomer, and that phosphorylation of ankyrin by the membrane cAMP-independent kinase [11] specifically depresses this binding to both tetramer and oligomer but not dimer spectrin. In addition, it is demonstrated that the cytoplasmic domain of protein 3 stimulates the binding of ankyrin to spectrin oligomers and enhances the rate of interchange between the various spectrin self-association states. The stimulatory effect of the 43-kDa protein 3 fragment is also shown to be inhibited by the phosphorylation of ankyrin. A model invoking an allosteric transition in spectrin is proposed to explain this long-range interaction linking protein 3 to spectrin oligomerization. Portions of this work have previously been presented in abstract form [12].

MATERIALS AND METHODS

Protein Purification

Spectrin was purified from low-ionic-strength extracts of fresh human erythrocyte membranes by gel-filtration chromatography on Sepharose CL-4B (Pharmacia) at 4°C in isotonic KCl buffer (10 mM Tris-HCl, 20 mM NaCl, 130 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 30 μM PMSF, pH 7.4) [13]. Extractions were performed at 37°C for 30 min in a 20-fold volume of extraction buffer (0.1 mM EDTA, 0.1 mM DFP, pH 9.3) or at 4°C for 36 hr by dialysis of the membranes against a 100-fold volume of extraction buffer.

Ankyrin was purified by ion-exchange chromatography (DE52 cellulose, Whatman) of a dialyzed 1 M KCl extract of spectrin-depleted fresh erythrocyte membranes [14], with minor modifications as described [15]. It was concentrated and dialyzed by vacuum dialysis (Biomolecular Dynamics, Beaverton, OR) initially against 0.1 M sodium phosphate, 1 mM EDTA, 100 μM PMSF, and 3% sucrose, pH 7.6, and then against isotonic KCl buffer containing 2% sucrose.

The cytoplasmic domain of protein 3 was solubilized from acetic-acid-stripped

erythrocyte membranes by incubation of the membranes for 45 min at 0°C with 200 μg/ml of alpha-chymotrypsin (Worthington Biochemical Corp.). The released peptides were purified by ion-exchange chromatography on DE52 cellulose (Whatman), followed by gel filtration at 4°C on a 2.5 × 100 cm agarose AcA44 column (LKB) as described [4,16]. For some preparations, a gel-filtration step using a 75 × 300 mm Bio-sil TSK250 HPLC column (Bio-Rad) at room temperature in 25 mM sodium phosphate, 1 mM EDTA, 1 mM sodium azide, 0.2 mM DTT, pH 7.5, was substituted for the AcA44 column. Either method of preparation yielded a mixture of 45–41-kDa peptides derived from the cytoplasmic domain of protein 3 and which retained ankyrin binding activity [16]. The purified peptides were concentrated by precipitation with 60% saturated ammonium sulfate at 0°C and dialyzed as described above for ankyrin.

The membrane-associated cAMP-independent kinase was prepared by extraction of fresh spectrin-depleted erythrocyte membrane vesicles for 30 min at 0°C with a 5 × volume of a 0.5 M NaCl buffer (5 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 60 μM PMSF, 0.1 mM DFP, pH 8.0) [11]. This extract was concentrated at 0°C by using an Amicon ultrafiltration apparatus with a PM-10 membrane and applied to a 2.5 × 100 cm column of G-100 Sephadex (Pharmacia). The column eluted with 10 mM Tris-HCl, 0.5 M NaCl, pH 8.0. The eluted fractions were assayed for kinase activity by incubating 50 μl of each fraction with 50 μl of a 1 mg/ml solution of casein (Sigma) in 20 mM Tris-HCl, 5 mM magnesium chloride, 150 mM KCl, pH 7.5, containing about 80 μCi of gamma-$[^{32}P]$-ATP (specific activity 100–250 dpm/pmole). The casein kinase activity eluted at 1.3–1.6 × Vo. (the void volume), and was used without further purification. Storage was at −70C.

Protein Iodination and Phosphorylation

Ankyrin or the 43-kDa fragment of protein 3 was labeled by reaction for 1 hr at 0°C with 1 mCi/mg of $[^{125}I]$-labeled Bolton-Hunter Reagent (Amersham). The buffer used in each case was isotonic KCl with 2% sucrose; the protein concentration was between 0.3 and 3.0 mg/ml. Generally, incorporation yields of 50–90% were achieved. After the reaction, the unreacted reagent was removed by gel filtration of the labeled proteins on a 1 × 15 cm column of G-25 Sephadex (Pharmacia) in isotonic KCl buffer at 0–4°C. For the binding studies, the appropriate amounts of labeled and unlabeled ankyrin were mixed to achieve the desired final specific activity. Separate inhibition experiments employing unlabeled ankyrin were used to established that the labeled material was fully active with respect to spectrin binding.

The phosphorylation of ankyrin (1 mg) was achieved by incubation at 0°C for 3–6 hr with a 1:8.5 dilution of the casein kinase preparation in isotonic KCl buffer without EDTA but containing 50 mM Tris-HCl, 2.5 mM Mg-ATP, 500 μCi of gamma-$[^{32}P]$-ATP, and 3% sucrose at pH 8.0. The unreacted ATP was removed from the protein by gel filtration with a 1 × 12 cm column of G-25 sephadex in isotonic KCl buffer with 3% sucrose. Endogenous phosphatase activity was inhibited by the inclusion of 0.5 mM NaF in the buffers for some experiments, although this was generally found to be unnecessary.

Endogenous phosphate was removed from the $[^{125}I]$-labeled ankyrin by incubation of a 0.4 mg/ml solution of ankyrin in isotonic KCl buffer, 3% sucrose, pH 7.6, overnight at 0°C with highly purified *E. coli* alkaline phosphatase at a ratio of 1:10 E:S (w/w). The alkaline phosphatase was a generous gift of Dr. J. Coleman; its purification and characterization have been described [31]. The enzyme had been fully loaded with zinc

during its preparation, and additional zinc was not included in the phosphatase experiments. The dephosphorylated ankyrin was used in the binding experiments without further purification after the addition of NaF to 50 mM.

Binding Studies

The interaction of spectrin with ankyrin and the 43-kDa protein 3 fragment was determined by incubation of the various components in isotonic KCl buffer at 0°C for 1 hr unless otherwise described in the text and legends. The extent of binding of ankyrin and/or the 43-kDa fragment to spectrin dimer, tetramer, and oligomer was determined by gamma counting of the appropriate Coomassie blue-stained and fixed bands after the separation of the various bound and free species using nondenaturing PAGE at 4°C, as previously described [4,9]. One modification used in the present series of experiments that differs from those previously performed using this technique is that the free concentration of ankyrin was taken directly from the value determined from the free ankyrin band on the gel, rather than as the difference between the amount of ankyrin loaded vs. that which could be accounted for as bound to the spectrin [4–6]. This technical change results in a systematic shift of the binding curves to lower concentrations of free ankyrin but does not change any of the conclusions of this or the previous studies. It does bring the quantitative values of the binding affinities as measured by this technique into better general agreement with the values determined by other techniques. Data and error analysis was performed by standard methods [33].

Other Procedures

Isotope counting was performed either by gamma counting of ^{125}I by using a Beckman Biogamma counter or by liquid scintillation counting (^{125}I and ^{32}P) in Optifluor (Packard) with a Beckman LS-230 liquid scintillation counter. Appropriate corrections for counting efficiency and quench were determined for these experiments using liquid scintillation counting. Double isotope counting was performed by counting ^{125}I in the "A" channel of the counter with the window limits of 0–3 while counting ^{32}P in the "B" channel with window limits of 4–10. These settings were found to provide nearly complete discrimination between those isotopes (see Results).

SDS-PAGE was performed on 5–15% gradient polyacrylamide gels by using a discontinuous buffer system [17]. Nondenaturing PAGE analysis was performed by using the method of Morrow and Haigh [18]. Protein determinations were by done by the method of Lowry et al. [19]. Rotary shadowed replicas of spectrin were prepared by low-angle carbon-platinum shadowing of solutions of spectrin dried from 70% glycerol in 0.2 M ammonium carbonate at pH 7.5 [20]. These replicas were visualized on a Philips EM300 electron microscope. Autoradiography was performed after drying of the polyacrylamide gels between cellulose sheets [18] by using Kodak XAR-5 film and fluorescent intensifying screens at −70°C. Quantitation of the amount of protein in Coomassie blue-stained bands on polyacrylamide gels was done by determination of the OD at 596 nm of the dye eluted from each band by 25% pyridine.

RESULTS
Ankyrin Binds Spectrin Tetramer and Oligomer With Enhanced Affinity

Previously, it has been noted that ankyrin binds to spectrin tetramer in preference to spectrin dimer when analyzed by nondenaturing PAGE [4–6] and that this phenomenon

must result from a cooperative coupling between the ankyrin binding site and the spectrin self-association site [4]. Ankyrin also binds to the oligomeric forms of spectrin more strongly than it does to spectrin dimer, with an affinity comparable to that for spectrin tetramer. These results are shown in Figure 1. Increasing amounts of [^{125}I]-labeled ankyrin were incubated with a fixed concentration of purified spectrin, and the distribution of bound ankyrin between the dimer, tetramer, and oligomeric forms of spectrin was determined after nondenaturing PAGE. For these experiments, the purified spectrin tetramer was concentrated to 4.6 mg/ml and then incubated at 37°C for 15 min to allow equilibration of the spectrin. At this concentration, approximately equal amounts of dimer, tetramer, and oligomeric species are formed [13,21]. The experiments with ankyrin were then carried out at 0–4°C in order to limit further changes in the distribution of spectrin between the various states. As is evident in the autoradiogram shown in Figure 1B, the radiolabeled ankyrin binds preferentially to the spectrin tetramer and oligomer forms; only when these species are nearly saturated does the ankyrin bind appreciably to spectrin dimer. These results are quantitated in Figure 1C. A double-reciprocal plot is shown; here the Kd is proportional to the slope. Note that the strongest binding is observed with spectrin tetramer (Kd = 41 ± 4 nM (2SD)) and that this value is similar to the binding observed for ankyrin to the various oligomeric species (Kd = 56 ± 11 nM (2SD)). (For the purposes of quantitation, the binding of ankyrin to all of the oligomeric spectrin forms (hexamer, octamer, etc.) has been measured as the total binding to all forms larger than tetramer and expressed as "oligomer" binding.) The binding to spectrin dimer is of lower affinity (Kd = 159 ± 7 nM (2SD)). Thus, both spectrin tetramer and spectrin oligomers display enhanced ankyrin affinity relative to spectrin dimer; the species with maximal affinity for ankyrin appears to be the tetramer.

The Cytoplasmic 43-kDa Domain of Protein 3 Enhances the Affinity of Ankyrin for Spectrin Oligomers

Chymotryptic digenstion of stripped erythrocyte membranes generates a series of protein 3 fragments between 45 and about 41 kDa. The predominant species are of about 43 and 41 kDa. All of these fragments arise from the cytoplasmic domain of the protein and all retain the ability to bind ankyrin in vitro [16]. These peptides are referred to collectively as the 43-kDa fragment. To determine the effect of this fragment on the ability of ankyrin to bind spectrin, solutions of spectrin at a constant concentration were incubated at 0°C with sub-stoichiometric amounts of either [^{125}I]-labeled ankyrin alone or with the 43-kDa fragment and [^{125}I]-labeled ankyrin. The molar amounts of ankyrin and 43-kDa fragment used in each experiment were approximately equivalent. The results of one experiment are shown in Figure 2. At the concentration of spectrin used in this experiment (0.3 mg/ml), most of it exists as dimer, and less than 2% is present as oligomeric forms larger than tetramer. This is evident from the Coomassie blue-stained gel shown in Figure 2a, lane 1, which contains spectrin with the 43-kDa fragment. The 43-kDa peptide alone has no influence on spectrin oligomers (data not shown). Ankyrin alone binds preferentially to the tetramer, as is evident by comparing the intensity of the autoradiogram (Fig. 2a, lane 7) with that of the Coomassie blue (lane 2; also cf. Fig. 1). The addition of the 43-kDa peptide and ankyrin to the spectrin produces a different pattern (lanes 3 and 8). When ankyrin loaded with the 43-kDa peptide binds to spectrin, the tetramer and oligomer spectrin bands become more diffuse, and additional radiolabeled ankyrin binds to the minor amounts of oligomer spectrin present in this experiment. Ankyrin alone (lanes 4 and 9) or ankyrin with the 43-kDa peptide (lanes 5
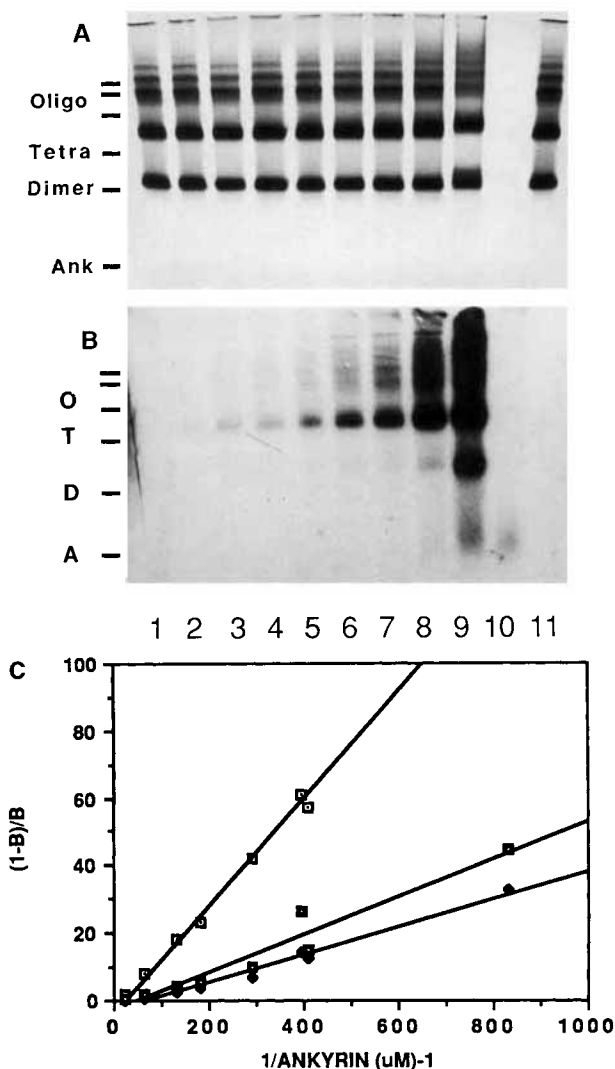
Fig. 1.   Ankyrin binds preferentially to spectrin tetramer and oligomer. **A:** A 10-μM solution of spectrin containing approximately equal amounts of dimer, tetramer, and oligomeric species was incubated at 0°C with increasing amounts of ^{125}I-labeled ankyrin. The Coomassie blue-stained nondenaturing gel is shown, demonstrating that there is no significant change in the relative abundance of the spectrin forms with increasing amounts of ankyrin. The free ankyrin (Ank) is nearly invisible on these Coomassie blue-stained gels. The ratio of ankyrin to spectrin (M/M) in each experiment was lane 1: 0.0 (no ankyrin); lane 2: 0.04; lane 3: 0.09; lane 4: 0.14; lane 5: 0.18; lane 6: 0.27; lane 7: 0.36; lane 8: 0.9; lane 9: 1.4; lane 10: ankyrin alone; lane 11: spectrin alone, as in lane 1. **B:** Autoradiogram of the gel shown above, demonstrating that the radiolabeled ankyrin binds initially to the spectrin tetramer and oligomer forms. Only when these are nearly saturated does appreciable binding to dimer spectrin become evident. The free ankyrin is also evident in this autoradiogram. **C:** Quantitiation of the amount of ankyrin bound to each spectrin form indicates that ankyrin binds spectrin tetramer (◆) four times more strongly than it binds dimer (□) spectrin. These data also suggest that ankyrin binds tetramer spectrin slightly better than it does oligomer (■) spectrin, although this difference is marginally significant. The Kd values determined from this double-reciprocal plot are 159 ± 7 nM for dimer spectrin, 41 ± 4 nM for tetramer spectrin, and 56 ± 11 nM for oligomer spectrin. (In this analysis, the bindings to all of the oligomer forms have been pooled and treated as a single entity. Error limits represent 2 standard deviations).
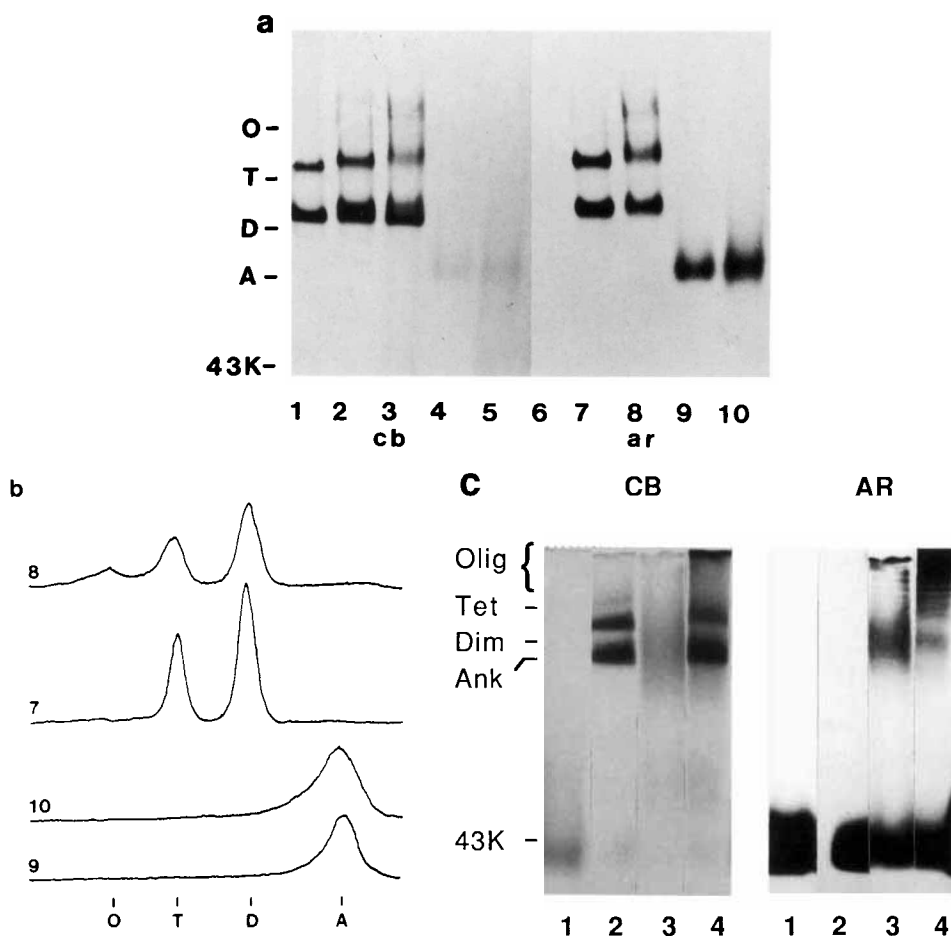
Fig. 2. The 43-kDa cytoplasmic fragment of protein 3 stimulates ankyrin binding to spectrin oligomers. Spectrin at a fixed concentration (0.06 μM) was incubated at 0°C with substoichiometric amounts of [125I]-labeled ankyrin (0.03 μM) in the presence or absence of the 43-kDa protein 3 fragment (11 μM). The ability of ankyrin to bind to the various spectrin species was then determined after nondenaturing PAGE as described in Figure 1. At the level of ankyrin used in this experiment, approximately 50% of the spectrin is bound to ankyrin. a: Lanes 1–5, Coomassie blue-stained gel; lanes 6–10, autoradiogram of the same gel, showing the distribution of the labeled ankyrin. The position of the various species on the gel is indicated at the left. Lanes 1 and 6 contain spectrin in the presence of the 43-kDa peptide. Lanes 2 and 7 contain spectrin and ankyrin with no 43-kDa peptide. Lanes 3 and 8 contain spectrin, ankyrin, and the 43-kDa peptide. Lanes 4 and 9 contain ankyrin alone, and lanes 5 and 10 contain ankyrin and 43-kDa peptide but no spectrin. b: Densitometric scans of the autoradiogram. Note the enhanced binding of ankyrin to the small amount of spectrin oligomer present when the solution contains the 43-kDa fragment. When the 43-kDa fragment binds to ankyrin, the ankyrin peak broadens slightly, but a separate band is not resolved (cf. lanes 9 and 10). c: The [125I]-labeled 43-Kda peptide also can be shown to bind directly to ankyrin and most strongly to high-molecular-weight spectrin-ankyrin complexes. There is no binding to spectrin directly. Shown on the left is the Coomassie blue-stained nondenaturing gel and on the right its corresponding autoradiogram. The total volume loaded onto each gel lane was 390 μl. The samples in each lane were lane 1, [125-I]-labeled 43-kDa peptide (1.5 μM); lane 2, a mixture of 0.2 μM spectrin with 0.6 μM [125I]-labeled 43-kDa peptide; lane 3, a mixture of 0.6 μM ankyrin with 0.6 μM [125I]-labeled 43-kDa peptide; lane 4, a mixture of 0.6 μM ankyrin, 0.2 μM spectrin, and 0.6 μM [125I]-labeled 43-kDa peptide. On the right is shown an autoradiogram of the same gel, except that lane 1 in the autoradiogram is from a gel loaded only with 0.6 μM [125I]-labeled 43-kDa peptide, so that it would be directly comparable to the other samples. The Coomassie blue-stained gel is presented with a larger load of the 43-kDa peptide so that the peptide can be more easily discerned. Note the enhanced binding of 43-kDa peptide to the high-molecular weight spectrin-ankyrin complexes and the absence of any direct interaction with spectrin.

and 10) displays no high-molecular-weight complexes which could account for the additional ankyrin comigrating with the spectrin oligomer bands, making it unlikely that the 43-kDa peptide is simply cross-linking two ankyrin molecules. This redistribution of ankyrin away from tetramer and into oligomeric spectrin is evident on densitometric scans of the autoradiograms, which detect the way ankyrin is distributing itself among the various spectrin species (fig. 2b).

The 43-kDa peptide itself also binds more strongly to spectrin-ankyrin complexes than it does to ankyrin alone. This observation is shown in Figure 2c. The 43-kDa peptide was labeled with [^{125}I], and then its binding to either spectrin or ankyrin alone or to a mixture of the two was examined by nondenaturing PAGE. In these experiments, the gel was electrophoresed only half of the normal time (24 hr vs. 48 hr) so that the relatively small 43-kDa peptide would remain on the gel. Under these conditions, there is incomplete separation of the ankyrin band from dimer spectrin. Nevertheless, it is clear that the 43-kDa peptide displays no interaction with spectrin alone (cf. lanes 1 and 2) and binds to ankyrin alone but introduces only a very slight shift in its mobility (lane 3; also compare lanes 9 and 10 in the densitometer scans in Fig. 2b). In the presence of spectrin and ankyrin, the amount of 43-kDa peptide binding to high-molecular-weight spectrin-ankyrin complexes is increased (lane 4), as would be expected if the 43-kDa peptide stimulated ankyrin binding to spectrin oligomers.

Ankyrin and the 43-kDa Fragment Enhance the Rate of Spectrin Dimer-Tetramer-Oligomer Interconversion

In the presence of ankyrin, and especially with both ankyrin and the 43-kDa fragment, the spectrin oligomer and tetramer bands become more diffuse when analyzed by nondenaturing PAGE [4]. This effect is particularly prominent at saturating levels of ankyrin and 43-kDa peptide (Fig. 3). One contribution to this band broadening appears to be an enhanced rate of dimer-tetramer-oligomer interchange that is induced by the bound ankyrin–43-kDa complex, as shown in Figure 3C. In this experiment, solutions of spectrin that were not at equilibrium with respect to the distribution of oligomer forms and which contained either buffer, saturating levels of ankyrin, the 43-kDa peptide, or both were incubated for various times at 30°C, after which the solutions were analyzed by nondenaturing PAGE at 4°C. In these experiments, since the initial and final amounts of spectrin tetramer were very similar, no significant changes in its abundance with time were noted. These results are not shown. Both spectrin dimer and hexamer relaxed to their equilibrium values with half-times bewtween 35 and 46 min, typical of the values determined previously for the interconversion of spectrin forms at this temperature [13,22]. The 43-kDa peptide when added alone had no significant effect on these rates. However, these time constants were reduced in the presence of ankyrin (10 min for dimer, 16 min for hexamer) and additionally foreshortened when the spectrin was saturated with both ankyrin and the 43-kDa peptide (7 min for dimer, 1 min for hexamer) (Fig. 3C). Thus, the ankyrin–43-kDa peptide complex must lower the activation barrier limiting the rate of interconversion between spectrin dimer, tetramer, and oligomers. This activation barrier has been previously estimated to be about 460 kJ/mol for the dissociation reaction of spectrin alone [22]. It also appears that ankyrin alone will perturb the interconversion rate for all forms of spectrin, while the effect of the 43-kDa–ankyrin complex is exerted primarily on the spectrin oligomer-to-tetramer interconversion. It is important to note that despite the enhanced rates of spectrin oligomer interconversion induced by the ankyrin–43-kDa peptide, the initial (t = 0) values for the amounts of hexamer, tetramer,
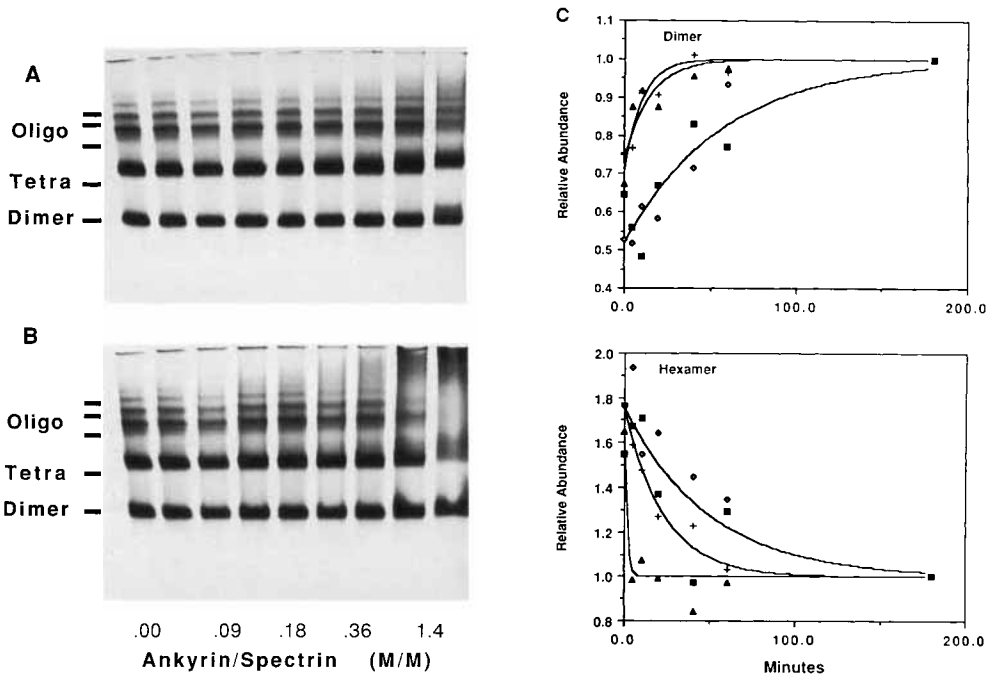
Fig. 3.   Ankyrin and the 43-kDa peptide enhance the rate of oligomer-tetramer-dimer interchange **A:** Coomassie blue-stained gel of spectrin incubated with increasing amounts of ankyrin, as in Figure 1A. The gel is reproduced here to facilitate a comparison with the effects of added 43-kDa peptide. **B:** Coomassie blue-stained gel showing the effects of incubating spectrin with increasing amounts of ankyrin that has been previously saturated with the 43-kDa cytoplasmic fragment of protein 3. The conditions for this experiment are otherwise identical to those shown in A. Note the broadening of the bands, which is most prominent for the oligomers and negligible for spectrin dimer. Despite the broadening and the obvious shift of the oligomers to larger forms, the total Coomassie blue dye bound by the dimer, tetramer, and the sum of the oligomers remains virtually unchanged in these experiments if the solutions are maintained at 0–4°C. **C:** The time course of the change in the dimer-tetramer-oligomer distribution of a solution of spectrin is shown. The spectrin solution was diluted ten-fold at 0°C to disturb the equilibrium and then incubated at 30°C for the periods indicated. Other conditions were as described in Figure 1. All solutions were identical, except for the presence of ankyrin, the 43-kDa peptide, or both. The ratio of 43 kDa : ankyrin : spectrin for these experiments was 6:4:1 (M/M). The time course for dimer and hexamer equilibrations are shown. Values are expressed as a ratio to their final value at 180 min. The solid curves represent nonlinear least-squares regression fits to the data. The symbols correspond to ($\diamond$), spectrin alone; ($\blacksquare$), spectrin with the 43-kDa peptide; (+), spectrin with ankyrin; and ($\blacktriangle$), spectrin with ankyrin and the 43-kDa peptide. Since the data for spectrin alone and for spectrin with the 43-kDa fragment are nearly indistinguishable, only a single composite curve fitted to these points is shown. Note the dramatic effect of the 43-kDa peptide in the presence of ankyrin on the time required for hexamer equilibration at 30°C (complete within 5 min).

and dimer present in the experiments shown in Figure 3C are similar. Thus, the rates still remain too slow to cause a significant redistribution of forms under the conditions of nondenaturing gel analysis if the solutions are maintained at 0–4°C.

Phosphorylation of Ankyrin Diminishes Its Affinity for Spectrin Tetramer and Oligomer, but Not Spectrin Dimer

Previously ankyrin has been determined to have as many as seven sites of covalent phosphorylation [5] and to be phosphorylated by several different kinases [5,23]. The

effects of phosphorylation by the membrane form of casein kinase on ankyrin's ability to bind to spectrin were investigated by comparing the amount of ^{32}P-labeled (phospho) ankyrin that bound to each oligomeric form of spectrin to the amount of ^{125}I-labeled ankyrin that bound in the same experiment. By using an isotope-ratio method in these experiments, potential sources of error due to difficulties in estimating the amounts of each spectrin species present on the gel, the specific activities or decay of the labeled proteins, or variations between different gels and in experimental conditions were eliminated. This is a consequence of the simple relationship that exists between the association constants (Ka) in a competitive binding experiment [9]. Specifically, for the isotope ratio experiment:

$$Ka(I)/Ka(P) \; = \; \frac{[I] \; bound}{[P] \; bound} \; \times \; \frac{[P] \; free}{[I] \; free} \tag{1}$$

In equation 1, [I] represents the cpm of the bound or free ^{125}I-labeled ankyrin; [P] represents similar values for the ^{32}P-labeled protein. To use this method, one must be able to discriminate clearly between isotopes in a single counting experiment. In the present experiment, nearly complete discrimination was achieved while maintaining counting efficiencies (relative to single isotope determinations) of 60% for ^{125}I and 86% for ^{32}P, as shown in Figure 4A. Conversely, less than 2% spillage of the ^{32}P counts were encountered in the ^{125}I channel, and less than 1% of the ^{125}I counts spilled into the ^{32}P channel.

The ratios of the association constants for iodinated (nonphosphorylated) and for phosphorylated ankyrin are shown in Figure 4B. Note that while both forms have similar affinities (ratio = 1.0) for dimer spectrin, the affinity of iodinated ankyrin for spectrin tetramer or oligomer is three to five times greater than that for the phosphorylated ankyrin. The ratio for spectrin oligomer is slightly less than for spectrin tetramer, but this difference is not statistically significant. When the 43-kDa peptide is bound to the ankyrin, the ratios are unchanged. In a separate experiment, in order to exclude the possibility that the incubation of ankyrin with the casein kinase damaged the protein, the ^{125}I-labeled ankyrin was incubated overnight at 0°C with a 1:10 (w/w) ratio of a purified solution of E. coli alkaline phosphatase (a generous gift of Dr. Joseph Coleman, Yale University). The binding experiment was then repeated. The alkaline phosphatase treatment enhanced the binding of the iodinated protein to tetramer by a factor of 1.3 ± 0.1 (data not shown), presumably due to the removal of residual phosphate on the iodinated ankyrin. Therefore, phosphorylation of ankyrin down-regulates its ability to bind spectrin tetramer and oligomers in preference to spectrin dimer, and this effect is not significantly diminished by the presence of the cytoplasmic domain of protein 3.

DISCUSSION

One pathway of signal transduction across the plasma membrane is likely to involve direct physical linkage between transmembrane proteins and soluble proteins of the cytomatrix [e.g., see 24–27]. A consequence of such linkage will be the assembly of potentially complex cytoskeletal structures in regions of the cell where there are high-affinity cytoplasmically directed membrane receptors [21,28]. The results reported here define a mechanism whereby the assembly of such structures may be actively and posttranslationally regulated. Three lines of evidence support this notion: (1) ankyrin
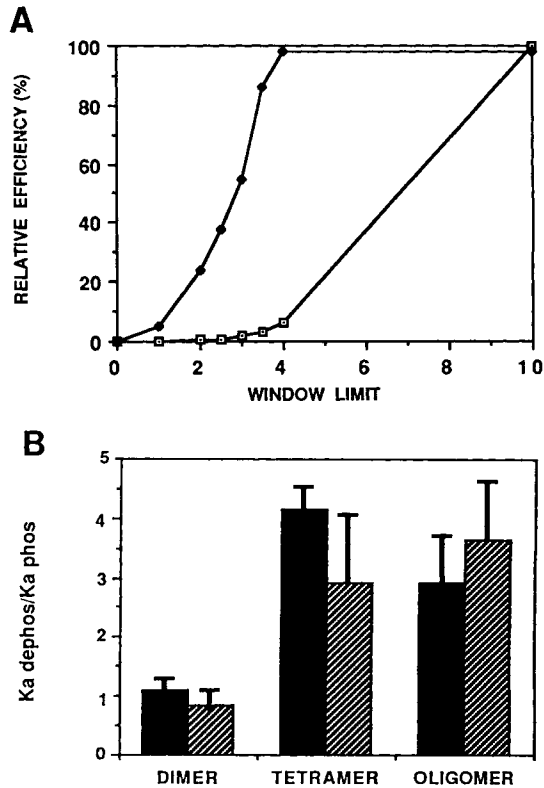
A

B

Fig. 4. Phosphorylation of ankyrin down-regulates its affinity for spectrin tetramer and oligomer. **A:** The effects of ankyrin phosphorylation on the Ka for ankyrin binding to spectrin was determined by comparing simultaneously the binding of ^{125}I-labeled (unphosphorylated) ankyrin to the binding of ^{32}P-labeled (phosphorylated) ankyrin. This technique required precise discrimination between ^{125}I and ^{32}P counts. Using samples of the radiolabeled ankyrin (^{125}I or ^{32}P) under the experimental conditions employed, the effects of different counting windows on the counting efficiency of the two isotopes were examined. The ordinate is expressed as counting efficiency relative to single isotope counting. Note that nearly complete discrimination between ^{125}I (closed symbols) and ^{32}P (open symbols) is possible. The window settings used for the experiment shown below were 0–3 for the iodine channel and 4–10 for the phosphate channel. The degree of overlapping counts with these settings was less than 1–2% for both isotopes. **B:** By using double isotope techniques, the ratio of the association constants (Ka) for unphosphorylated and phosphorylated ankyrin binding to each spectrin species could be determined directly. The data shown are from 7 separate determinations. Note that while there is no significant effect of phosphorylation on the binding of ankyrin to dimer spectrin, the unphosphorylated ankyrin binds spectrin tetramer and oligomer three to four times better than phosphorylated ankyrin. The presence of the 43-kDa peptide has no significant effect on this regulation. Solid bars represent ankyrin. Hatched bars represent ankyrin with the 43-kDa peptide. Error bars are SD.

binds with greatest affinity to spectrin tetramer and oligomer; (2) the cytoplasmic domain of protein 3 enhances the binding of ankyrin for spectrin oligomer, but not spectrin tetramer; and (3) phosphorylation of ankyrin by a cAMP-independent kinase inhibits both effects. In addition, these results also suggest that the interconversion rate between the different states of spectrin self-association will be enhanced in the presence of ankyrin and the cytoplasmic domain of protein 3, suggesting that in situ the erythrocyte membrane skeleton may be more fluid than anticipated from in vitro studies of the isolated proteins.

It is conceivable that the enhanced binding of ankyrin to the oligomeric forms of spectrin in the presence of the 43-kDa fragment could arise from a secondary, albeit weak, interaction between the ankyrin or the 43-kDa molecules themselves. Presumably, such an interaction would be too weak to measure with the isolated protein (cf. Fig. 2) but would contribute to the enhanced affinity of ankyrin when one or more ankyrins were already bound to a spectrin tetramer or oligomer. However, it would be difficult for such a mechanism to account for the enhanced rate of dissociation of the oligomeric complexes in the presence of ankyrin and the 43-kDa peptide at 30°C, since it is unclear how any mechanism which did not perturb directly the activation barrier characteristic of the spectrin-spectrin linkage could enhance the rate of oligomer interchange. Therefore, an allosteric linkage between these three proteins must be considered to explain the results reported here.

Spectrin is a highly asymmetric molecule composed of repetitive 106-residue subunits [29]. Given that the ankyrin binding site and the site of heterodimer polymerization are separated by nearly 30 kDa (approximately 3 repetitive units), what mechanisms might account for such a long-range effect? While conformational rearrangements along the axis of the spectrin heterodimer cannot be excluded, it is more likely that changes in the flexibility of spectrin, perhaps involving some sort of ''hinge'' region, might be involved. This hypothesis is depicted in Figure 5 and is supported by several observations. It is likely that the formation of the tetramer from the heterodimer involves a rearrangement of existing noncovalent bonds between the alpha and beta subunits, since no pairs of noncovalently associated peptides have been identified in tryptic digests of the tetramer that are not also present in the dimer [9]. This rearrangement of existing noncovalent bonds has been postulated to account for the high activation barrier required for tetramer (or oligomer) formation [13,20,22]. It has also been noted that the formation of oligomers appears to be characterized by a reduced association constant compared to that for tetramer formation [21]. One explanation of these observations would postulate a conformationally labile ''hinge'' region near the COOH terminus of the spectrin beta subunit (and/or the alpha subunit) which undergoes increasing degrees of strain as it deforms to accommodate the formation of tetramers and oligomers. If ankyrin binds at or near this ''hinge,'' then it might easily alter the strain about this site and thereby

Fig. 5. Hypothetical mechanism of the spectrin–ankyrin–43-kDa peptide allosteric interaction. A: Rotary-shadowed carbon-platinum replicas of purified spectrin demonstrate that the two subunits of the heterodimer are often loosely joined and may be the most conformationally heterogeneous at the end involved in dimer-tetramer-oligomer interactions. Top: spectrin heterodimer (for the dimer, the end involved with tetramer formation cannot be determined with certainty from these experiments). Middle: spectrin tetramer. Bottom: spectrin hexamer. Bar represents 100 nM. B: Schematic depicting the apparent conformation of three of the spectrin molecules shown in A. The arrowheads mark the sites of putative ankyrin binding (20 nM from the site of self-association) [14]. Note that this site appears to often be a point of contact between the two subunits and that the net angularity across this site becomes more acute as one progresses from the dimer to the hexamer. C: One mechanism by which ankyrin binding might influence the self-association reaction of the spectrin heterodimers could involve alterations in the conformation and flexibility of a ''hinge'' region at or near the ankyrin binding site. Such a conformationally labile site might explain the high protease sensitivity of the ankyrin binding domain [9] as well as the ''action at a distance'' which appears to characterize the ankyrin effect. The 43 kDa is envisioned to act by inducing a conformational change in ankyrin, which secondarily alters its effects on the conformation and flexibility of the putative spectrin ''hinge.'' Phosphorylation of ankyrin might act by a similar mechanism or by reducing the affinity of a binding site which is utilized only in tetramer and oligomer spectrin.
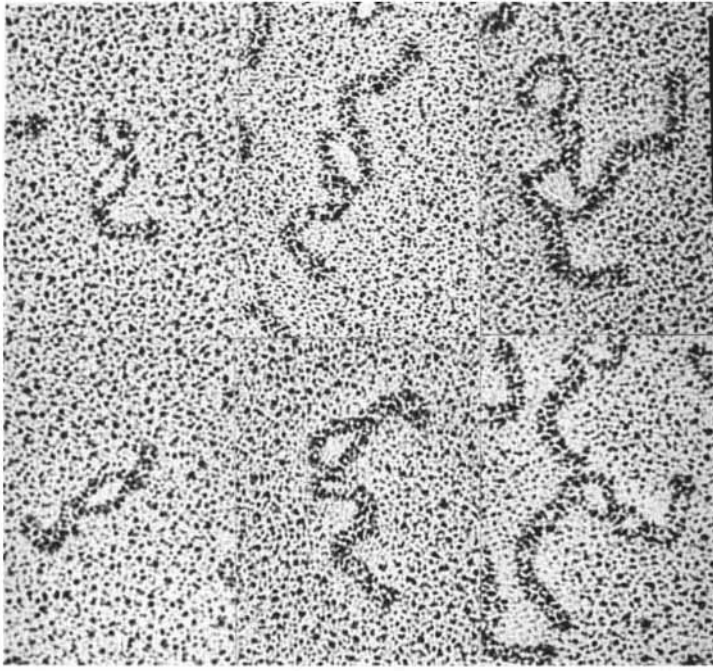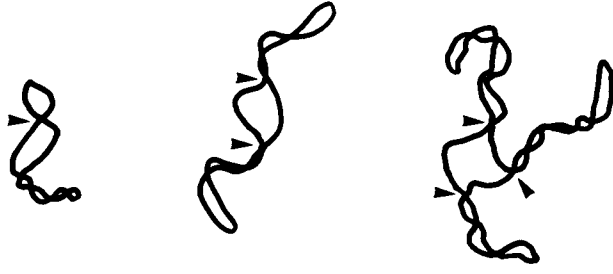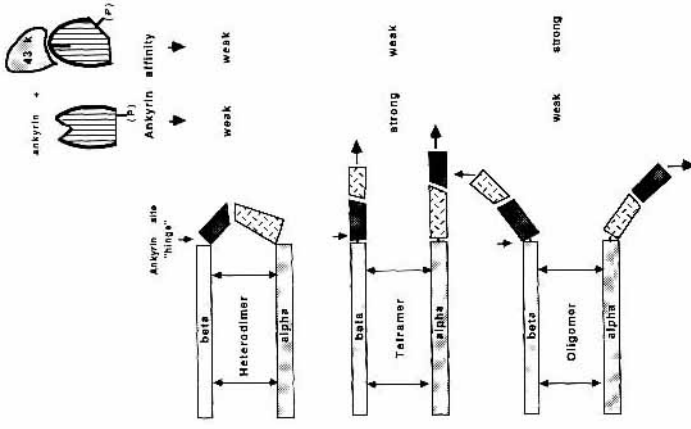
Figure 5.

regulate both the rate and extent of oligomer formation. Consonant with this notion is the observation that at the postulated site of ankyrin binding (fig. 5B) [14], rotary-shadowed replicas of spectrin often show the two subunits to be joined while beyond this site on the side toward the self-association site they are usually widely separated. Conversely, the 43-kDa peptide and ankyrin phosphorylation presumably transmit their effects via a conformational rearrangement in ankyrin. This hypothesis is summarized in Figure 5C.

Regardless of the mechanistic details, these results extend our understanding of a molecular interaction whereby surface receptors and the cortical cytoskeleton may be functionally coupled and controlled. It is likely that such interactions exist in other cells. They may be of importance for the regulation of cell shape and growth by the extracellular matrix [25] or for the polarized assembly of receptors and the cytoskeleton [28]. It will also be of interest to determine if there is a reciprocal effect of the cytoskeleton on the function of certain surface proteins, such as the Na/K ATPase of renal epithelia [30,32].

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