Heterogeneous phosphorylation of erythrocyte spectrin β chain in intact cells

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Human erythrocyte spectrin is an $\alpha\beta$ heterodimer which forms tetramers by self-association. This association involves the N-terminal region of the α chain and the C-terminal region of the β chain. The latter contains a cluster of four phosphorylation sites (one phosphothreonine and three phosphoserine residues). The role of this phosphorylation is as yet unknown. We show in this paper that the spectrin β chain occurs in the cell in subpopulations differing in the degree of occupancy of their phosphorylation sites: ^{32}P peptide maps obtained by 2-nitro-5-thiocyanobenzoic acid (NTCB) cleavage revealed the presence of six components with apparent molecular masses of 17.5 kDa, differing in their isoelectric points; this is most simply interpreted

as reflecting the presence of six exchangeable phosphorylation sites in the spectrin β chain, rather than four as had been supposed. When the $\alpha\beta$ dimers were partly dissociated by urea, the most highly phosphorylated fraction of the β chain was found in the undissociated dimers. This high specific activity in the undissociated dimer reflected multiple phosphorylated sites, as revealed by NTCB cleavage. The dephosphorylation or the hyperphosphorylation of spectrin β chains did not modify the equilibrium between dissociated and undissociated spectrin dimers in the presence of urea. However, the data revealed the existence of two spectrin dimer populations in respect to phosphate turnover and spectrin dimer dissociation.

INTRODUCTION

It is now well established that the shape and the mechanical properties of mammalian erythrocytes depend largely on a submembranous protein skeleton made up of spectrin, actin, protein 4.1, protein 4.9 and some other proteins such as adducin, tropomyosin, myosin and tropomodulin (Bennett, 1990). This structure results from multiple non-covalent interactions between the different proteins. Although the structure and the organization of the skeleton and the putative associations between the components have been investigated in reasonable detail, there is little understanding about post-translational regulation of the protein–protein interactions. Among the numerous factors that may serve as regulators of skeletal organization, phosphorylation is one that may play an important part. In the erythrocyte, all of the components, except actin, of the membrane skeleton, including the proteins involved in its attachment to the lipid bilayer (such as ankyrin and protein band 3), are phosphoproteins, and thus substrates for several erythrocyte protein-kinases (Boivin, 1988).

Recent observations based on studies *in vitro* strongly suggest that phosphorylation may modify interactions between proteins, leading in all cases to a reduced affinity. This was the case for the association of phosphorylated band 2.1 with spectrin tetramers (Lu et al., 1985; Cianci et al., 1988), and with band 3 (Soong et al., 1987), of phosphorylated protein 4.9 with F-actin (Husain-Chishti et al., 1988) and binding of phosphorylated band 4.1 to the membrane (Danilov et al., 1990; Chao and Tao, 1991) and its ability to promote spectrin–actin association (Eder et al., 1986; Husain-Chishti et al., 1988; Ling et al., 1988). But up to now, the phosphorylation of spectrin, which is the major structural component of the skeleton, has remained enigmatic (Ungewickell and Gratzer, 1978; Anderson and Tyler, 1980; Lecomte et al., 1982). Spectrin is composed of two flexible chains, α and β (calculated molecular mass, 280 and 246 kDa respectively) which

are laterally associated to form the heterodimer. In the cell, spectrin occurs as a tetramer, made up of two heterodimers joined head to head. This association involves the α -chain N-terminus (αI domain) and the β -chain C-terminus (βI domain). Four exchangeable phosphoryl groups of spectrin (three phosphoserine and one phosphothreonine residues), are located in a cluster at the C-terminal end of the β chain (Harris and Lux, 1980). However, spectrin β chain phosphorylation does not seem to affect tetramer formation (Ungewickell and Gratzer, 1978; Anderson and Tyler, 1980; Harris et al., 1980) or binding to ankyrin (Anderson and Tyler, 1980; Lecomte et al., 1982; Lu et al., 1985).

We report in this paper that the spectrin β chain in the cell is heterogeneous in respect of phosphorylation. We also show that the turnover of phosphates is linked to the presence of a dissociation-resistant population of dimers in urea solutions. Finally, we consider the presence of two populations of dimers in the membrane: one with a great intradimer stability and highly exchangeable phosphates; the other with a low turnover of phosphate, easily dissociated by urea.

MATERIALS AND METHODS

Spectrin preparation

Spectrin was extracted by incubating haemoglobin-free ghosts (Dodge et al., 1963) for 30 min at 37 °C in low-ionic-strength buffer [0.3 mM sodium phosphate buffer, pH 8.0, 0.1 mM phenylmethanesulphonyl fluoride, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol (2-ME)].

Separation of α and β chains

Spectrin α and β chains were essentially separated as described by Yoshino and Marchesi (1984) except that a low-pressure ion-

Abbreviations used: DMS, dimethylsulphurimide; 2-ME, 2-mercaptoethanol; NTCB, 2-nitro-5-thiocyanobenzoic acid.

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exchange Mono Q column (Pharmacia) was used (Lecomte et al., 1990).

Spectrin extracts (2–3 mg) were incubated in 3 M urea/10 mM 2-ME/1 mM EDTA/20 mM Tris/HCl, pH 8.0, for 60 min at 0 °C, and applied to a column equilibrated with the same buffer. The elution was performed with a linear gradient of NaCl (0.25–0.40 M) at a flow rate of 1 ml/min.

Phosphorylation of spectrin in cells

^{32}P -labelled spectrin was obtained essentially as described by Anderson and Tyler (1980). Washed erythrocytes were incubated in isotonic solution, comprising 130 mM NaCl, 3.7 mM KCl, 2.4 mM MgCl_2 , 1.2 mM CaCl_2 , 25 mM NaHCO_3 , 1 mM adenosine, 10 mM glucose, 1 mM aprotinin, 0.1 mg/ml streptomycin and 0.1 mg/ml penicillin, for 25 h at 37 °C in 5% CO_2 atmosphere. Neutralized $\text{Na}_2\text{H}^{32}\text{PO}_4$ (0.5 mCi) (specific radioactivity 200 mCi/mmol; Amersham) was added to 1 ml of packed cells.

The radioactivity of the elution fractions was determined using an Intertech SL 3000 scintillation counter.

Spectrin cross-linking

Spectrin was cross-linked by dimethylsuberimidate (DMS) (Fluka) as previously described (Swaney and O'Brien, 1978; Calbert et al., 1980).

The cross-linking reagent (20 mg) was dissolved in 0.1 M triethanolamine, pH 8.5. This solution was added to 10 vol. of the spectrin solution (40 $\mu\text{g}/\text{ml}$) in the presence or absence of 3 M urea. The reaction was carried out overnight at 4 °C with agitation and was stopped by addition of SDS. The DMS was made up immediately before use.

Spectrin hydrolysis at cysteine residues

As previously described (Speicher et al., 1982), lyophilized salt-free spectrin subunits were dissolved in 200 mM Tris/HCl/7.5 M guanidinium chloride/1 mM EDTA (pH 8.0). Reaction was initiated by addition of NTCB. After 1 h at room temperature, the pH was raised to 9.0 by addition of 1 M Tris base. The solution was incubated at 37 °C overnight and the reaction was stopped by adding 2-ME (25 mM final concentration).

Salts were removed by dialysis before two-dimensional PAGE by O'Farrell's method (1975), as modified by Speicher et al. (1982), except that ampholines, pH range 3.5–5, were used instead of ampholines pH range 5–7.

Polyacrylamide gel electrophoresis

SDS/PAGE was performed as described by Laemmli (1970). Gels were stained with Coomassie Blue (Fairbanks et al., 1971). Densitometric tracings were performed using an UltroScan XL laser densitometer (Pharmacia).

RESULTS

Isolation of α and β spectrin subunits by anion-exchange chromatography on a Mono Q column

After incubation in 3 M urea, spectrin extracts were applied to a Mono Q column and fractionated with a linear salt gradient

(0.25–0.4 M). A typical peptide elution profile is shown in Figure 1. As previously reported (Lecomte et al., 1990), the minor proteins present in crude spectrin extracts (adducin, protein 4.1, protein 4.9, actin and glyceraldehyde-phosphate dehydrogenase) were not retained on the column. The β chain was eluted first at 0.28–0.30 M NaCl, followed by the α chain, which emerged in the two succeeding peaks. SDS/PAGE revealed that the β chain was pure, whereas the α chain was eluted with contaminating β chain (Figure 2a). As previously reported, the ratio [β chain/ (α plus β chains)] varied throughout the elution profile, with minimal contamination at the top of the second peak (Lecomte et al., 1990).

Figure 1 Elution profile of ^{32}P -labelled crude spectrin extract obtained from chromatography on a Mono Q column in the presence of 3 M urea, showing absorbance (right ordinate) and radioactivity (left ordinate)

Abbreviations: PA, peak A containing minor proteins; PB, peak B containing pure β -spectrin chain; PC and PD, peaks C and D containing spectrin α chain.

Figure 2 SDS/PAGE of spectrin before and after treatment with DMS

A 5% (w/v) acrylamide gel was used: lane 1, crude spectrin extract without urea; lane 2, crude spectrin extract in the presence of 3 M urea; lane 3, peak of isolated β chain; and lanes 4 and 5, isolated α chain from the top of peak D. (a) Before treatment with DMS and (b) after treatment with DMS. Abbreviation: HMM, high-molecular-mass species.

Chromatography on a Mono Q column of spectrin dimers (purified as described by Ungewickell and Gratzner, 1978) instead of crude spectrin extracts did not improve the separation or the purity of the products. Furthermore, fractions containing the α chain, when re-chromatographed on the Mono Q column, did not show improved separation.

The presence of β chain in the α chain zone could be due to undissociated dimer or retarded elution of free β chains. To answer this question, the different spectrin species isolated from the Mono Q column were analysed by SDS/PAGE after chemical cross-linking with DMS (Figure 2b). As a control, reaction of spectrin dimer with DMS in the absence of urea led to the appearance of high-molecular-mass species with the concomitant disappearance of SDS-dissociated α and β subunits. When cross-linking was carried out in the presence of 3 M urea, the high-molecular-mass species were still generated, but single α and β chains could also be seen in the SDS/polyacrylamide gel. Exposure of pure β chain dissolved in 3 M urea to the bifunctional reagent did not result in formation of high-molecular-mass species. In contrast, material from the α -chain peak prepared in 3 M urea gave rise to high-molecular-mass species which migrated at the position of the cross-linked dimer. More obviously, the amount of β chain was concomitantly strongly diminished, leading to a decreased [β chain/(α plus β chains)] ratio. These results indicate the presence of undissociated spectrin subunits co-eluted with spectrin α chain in 3 M urea.

Separation of spectrin subunits in 3.5 and 4 M urea

To optimize α - and β -subunit dissociation, chromatography was performed in the presence of different urea concentrations (2.5–4 M). In 3.5 M urea, the yield of pure β chain reached its maximum value of 66 % (Table 1) but the α chain remained contaminated.

Moreover, the elution of the α chain was progressively shifted to lower salt concentrations with increasing urea concentrations, and the trailing edge of the β -chain zone became contaminated with α chain. This feature was so pronounced in 4 M urea that all fractions of the β -chain peak contained small amounts of α chain, whereas the α chain remained contaminated with β chain.

Table 1 Yield of spectrin dissociation expressed as a percentage of eluted pure β chain

Spectrin was labelled in the presence of ^{32}P after incubation for 20 h and 6 h. Percentages of pure β chain are expressed as $100 \times (\text{amount of pure } \beta \text{ chain})/(\text{total eluted } \beta\text{-chain ratio})$. The percentages are the mean values obtained from n different experiments.

	Pure β chain eluted (%)			
	Urea concentration (M) ... 2.5	3.0	3.5	4.0
Incubation for 20 h				
Standard spectrin	32 $\pm$ 6 ($n = 4$)	52 $\pm$ 4 ($n = 6$)	66 $\pm$ 8.5 ($n = 5$)	0
Dephosphorylated spectrin	—	47 ($n = 2$)	—	—
Incubation for 6 h				
Standard spectrin	—	50 ($n = 2$)	—	—
Hyperphosphorylated spectrin	—	45 ($n = 2$)	—	—

Figure 3 SDS/PAGE (10% acrylamide) of fractions corresponding to peak A (PA) and crude spectrin (C), stained with Coomassie Blue (CB) and the corresponding autoradiogram (A)

Purification of labelled β chain

Labelled spectrin was obtained after incubation of washed erythrocytes in the presence of ^{32}P . The fractionation profile of labelled spectrin on Mono Q (Figure 1) was similar to that obtained with the unlabelled protein (Lecomte et al., 1990).

The contaminant proteins present in crude spectrin extracts and not retained on the column (Figure 2a), including proteins 4.1, 4.9 and adducin, were highly labelled. Labelling of protein 4.9 and adducin was particularly marked with high radioactivity in the autoradiograms corresponding to weakly stained zones (Figure 3). The specific radioactivity of the β chain increased across the elution profile, the most strongly labelled molecules being eluted last (Figure 4). More interestingly, the highest specific radioactivity was found in the part of the β chain co-eluting with α chain, corresponding therefore to the undissociated spectrin dimer. This was true at all urea concentrations examined (Table 2). Maximal specific radioactivity in the undissociated dimer corresponded to the highest degree of fractionation of α and β chains, which occurred at 3.5 M urea.

Peptide maps from phosphorylated β -chain peptides

Phosphorylated pure β chain (peak B) and undissociated phosphorylated dimer (peak D) recovered from the Mono Q column were hydrolysed at cysteine residues using NTCB. Autoradiography of the slab gels obtained after two-dimensional electrophoresis showed three sets of phosphorylated peptides

Figure 4 Electrophoretic analysis of the elution profile shown in Figure 1

Fractions corresponding to peaks B, C and D (PB, PC, PD respectively) were electrophoresed on SDS/6% polyacrylamide gel. Upper panel: staining with Coomassie Blue; lower panel: corresponding autoradiogram.

Table 2 Specific radioactivity of pure (i.e. free) β chain and β chain in the undissociated dimer (bound β chain) at different urea concentrations

Urea concn. (M)	Specific radioactivity (c.p.m./ μ g)		
	Free β chain	Bound β chain	Bound/free
2.5	5.2	14.6	2.8
3.0	5.2	15.0	2.9
3.5	5.7	33.5	5.9

with apparent molecular masses of 35, 21 and 17.5 kDa (Figure 5). The predominant labelled zones were at 21 and 17.5 kDa, each having components with different isoelectric points. The group of peptides of 21 kDa were poorly resolved because of their very close isoelectric points. In contrast, those at 17.5 kDa revealed a greater number of resolved components. NTCB cleavage of the first β -chain fractions to elute from the column, which were poorly phosphorylated, gave rise to two distinct labelled spots, and a third additional more acidic fragment was generated by the pure β chain [Figures 5(a) and 5(b)] which eluted later. The peptide maps generated by the undissociated dimer, contained up to six spots, three of which were more acidic than any derived from simple β chains (Figure 5d).

Figure 5 Autoradiograms of two-dimensional peptide maps obtained after NTCB cleavage

(a) First fractions of peak B elution (corresponding to pure β chain), (b) last fractions of peak B elution, (c) first fractions of peak D elution (corresponding to enriched α chain) and (d) last fractions of peak D elution. Abbreviation: IEF, isoelectrofocusing.

Dissociation of dephosphorylated or hyperphosphorylated spectrin in the presence of 3 M urea

Dephosphorylated spectrin was obtained after metabolic depletion of erythrocytes. Erythrocytes were first labelled in the presence of ^{32}P , then they were incubated at 37 °C in metabolic depletion conditions (without glucose). After 24 h of depletion, spectrin did not contain any labelled phosphate. The fractionation profile of dephosphorylated spectrin on a Mono Q column in the presence of 3 M urea was similar to that given with spectrin obtained in standard conditions (fresh erythrocytes). The yield of pure β chain obtained from dephosphorylated spectrin and from standard spectrin was 47% and 52% respectively (Table 1). Hyperphosphorylated spectrin was obtained after incubation of erythrocytes in the presence of okadaic acid, an inhibitor of phosphatases (Cohen et al., 1990). Erythrocytes were first incubated in the presence of ^{32}P for 3 h, then for 3 h more in the presence of 1 μM okadaic acid. Crude spectrin extracts were twice as strongly labelled in comparison with spectrin extracts from erythrocytes incubated without okadaic acid.

As observed with dephosphorylated spectrin, the yield of dissociation of hyperphosphorylated spectrin did not change, as expressed by the percentage of eluted pure β chain (hyperphosphorylated spectrin 45%, $n = 2$; standard spectrin 50%, $n = 2$). After 6 h of incubation in the presence of ^{32}P , either with or without okadaic acid, free β chain was poorly labelled with similar specific radioactivities. In contrast undissociated dimer was highly labelled, particularly undissociated dimer obtained after incubation of erythrocytes in the presence of

okadaic acid. In the presence of phosphatase inhibitor, the specific radioactivity of undissociated dimer is twice as high as that of undissociated dimer obtained in the absence of okadaic acid and eight times higher than the specific radioactivity of free β chain.

NTCB-cleaved peptide maps obtained from hyperphosphorylated spectrin after a 6-h incubation were similar to those obtained after a 20-h incubation (results not shown).

DISCUSSION

We have demonstrated the persistence of undissociated spectrin dimers at urea concentrations up to 3.5 M. This appears to be related to the phosphorylation state of the β chain.

It has been determined that the spectrin dimer possesses an average of four covalently bound exchangeable phosphoryl groups (three phosphoserine residues and one phosphothreonine residue) located in a cluster close to the β -chain C-terminus (Harris et al., 1980). All of these could be labelled in the intact erythrocytes by incubation *in vitro* in the presence of [32 P]P_i (Anderson and Tyler, 1980). Harris et al. (1980) have also shown that, at any time, approx. 10% of these spectrin phosphate sites are free, but they did not investigate whether the occupancy of the four phosphorylation sites in a spectrin preparation is random or whether there exists a population of molecules with no, or few, phosphoryl groups.

Chromatography of partially dissociated spectrin dimers showed that the spectrin molecules are not randomly phosphorylated. Our argument that there are distinct populations differing in extent of phosphorylation depends on full or nearly full phosphorylation of spectrin overall, such as results from the labelling method described by Anderson and Tyler (1980). These authors have clearly shown that maximal spectrin labelling was obtained after incubation for 20 h with [32 P]P_i. Peptide maps obtained by NTCB cleavage revealed a set of phosphorylated peptides having the same molecular mass (17.5 kDa) and different isoelectric points, consistent with differing phosphate contents.

Examination of mutant β chains, truncated at their C-terminal ends, which are found in forms of hereditary elliptocytosis has shown that all exchangeable sites are located on the C-terminal side of residue 2046 (Tse et al., 1991; Lecomte et al., 1992), i.e. after the last cysteine residue (at position 1212). Thus the smallest peptide, with a calculated molecular mass of 16.4 kDa, resulting from cleavage at this cysteine residue, must contain all the phosphorylatable sites. The multiply labelled components derived from this fragment (apparent molecular mass 17.5 kDa) with different isoelectric points, thus presumably differ from each other only in phosphate content.

Peptide maps revealed the presence of at least six different labelled spots at 17.5 kDa. These spots cannot result from a different combination of occupied and unoccupied phosphorylation sites if the total number is only four. The presence of more than four exchangeable phosphates per spectrin dimer can be inferred: each added phosphate would be expected to cause a similar drop in isoelectric point. The value of four phosphates per dimer would then represent an average for all the populations with different phosphate contents.

Phosphorylation of spectrin involves several kinases including (i) a membrane-bound casein kinase (type I) (Boivin, 1988), that only phosphorylates serine residues, and (ii) a cytosolic casein kinase (type II) that phosphorylates serine as well as threonine residues (M. C. Lecomte, unpublished work). In addition, Mische and Morrow (1990) have recently reported a calcium-calmodulin-dependent kinase which actually phosphorylates

serine and threonine residues. According to Tuazon and Traugh (1991) the recognition sequences presently known for casein kinases I and II required the presence of acidic residues either on the N-terminal side of serine for casein kinase I or on the C-terminal side of serine and threonine for casein kinase II. Therefore the primary sequence of the C-terminal end of the β chain (Winkelmann et al., 1990) offers more than six potentially phosphorylatable sites for both enzymes. Moreover, a consensus sequence is also available for the calcium-calmodulin-dependent protein kinase (Colbran et al., 1989).

The phosphorylation results reported here can be linked finally to the presence of a dissociation-resistant population of dimers in urea solutions. In the presence of 3 M urea, the spectrin dimer pool extracted at 37 °C can be divided into two equal populations. The first population can dissociate into α and β chains (the β chain being poorly phosphorylated) and the second one is a dissociation-resistant population. This spectrin dimer population contains highly exchangeable phosphates, as indicated by either hyperphosphorylation or dephosphorylation procedures. Inhibition of phosphatases by okadaic acid increases the phosphorylation status of the normally phosphorylated spectrin and has no effect on the poorly labelled β -chain pool. Therefore, okadaic acid emphasized the differences in the phosphorylation processes between the two populations of spectrin observed under standard conditions but did not modify the spectrin dimer dissociation *in vitro*. These results suggest the presence of two spectrin dimer populations in the membrane, with regard to phosphate turnover in the cell and spectrin dimer stability. One population of spectrin dimer has a conformational structure or another unknown specificity, exposing the phosphorylatable sites and stabilizing the α - β interchain association, in contrast with the other spectrin dimer population, displaying a low turnover of phosphates and being dissociable in denaturant conditions *in vitro*. The dissociation yields of spectrin dimer pool in 3 M urea under different conditions of phosphorylation has pointed out the consistency of two equilibrated populations (50% dissociated spectrin versus 50% undissociated). This feature is reminiscent of the status of the ankyrin-binding sites of spectrin, since only one site in the tetramer is able to bind ankyrin (Weaver et al., 1984). The hypothesis, that in the membrane the phosphorylation status of each dimer which composes a tetramer is also non-equivalent, has to be confirmed by other studies.

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Received 2 March 1993; accepted 15 April 1993