A cyclic AMP-dependent phosphorylation of spectrin dimer

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In contrast to the properties of spectrin obtained from [^{32}P]phosphate-labeled red cells, purified spectrin dimer could be phosphorylated by a cAMP-dependent protein kinase from bovine heart. Both spectrin bands were phosphorylated. Spectrin band 2 contained in addition to autophosphorylated peptides several phosphopeptides that were distinct from autophosphorylated ones. The cAMP-dependent phosphorylation of spectrin band 1 was modulated by reducing agent and the concentration of spectrin. At high concentrations spectrin band 2 was predominantly labeled. The cAMP-dependent phosphoform of spectrin band 2 had a p*I* slightly higher than that of autophosphorylated spectrin band 2, but lower than that of ankyrin.

Erythrocyte *Cytoskeleton* *Phosphorylation* *Spectrin* *cAMP*

1. INTRODUCTION

Purified spectrin dimer from human red cells contains 4 phosphates within a cyanogen bromide fragment of 19 kDa at the carboxy-terminal end of band 2 [1]. The same terminal fragment of band 2 is labeled following in vitro phosphorylation of purified spectrin dimer with [γ-^{32}P]ATP alone (autophosphorylation) [2,3] or in the presence of a cAMP-independent protein kinase [2–4]. Hence, spectrin appears unique in the sense that only one of its subunits is phosphorylated. In contrast, several groups reported that spectrin is cAMP-dependently phosphorylated in ghosts and labeled in spectrin band 1 and band 2 [5,6]. More recently, authors in [7] demonstrated a cAMP-dependent phosphorylation of spectrin in cytoskeletons, but not in purified spectrin dimer alone. These findings reveal an unresolved discrepancy that has not drawn enough attention in recent years [8]. To investigate the phosphoryl acceptor capacities, spectrin phosphorylation was studied in the presence of a cAMP-dependent protein kinase from bovine heart which has a wide substrate specificity [9].

Abbreviation: ATP[γ^{35}S], adenosine 5′-[γ-[^{35}S]thio]-triphosphate

2. MATERIALS AND METHODS

2.1. Purification of spectrin

Human red blood cells were freed from white cells [10] and membranes were isolated and treated with diisopropyl fluorophosphate as in [11]. Spectrin was extracted from membranes as a dimer [12] and purified on CL-4B Sepharose (Pharmacia, Uppsala) equilibrated essentially as in [13], except that the buffer contained in addition 0.01 mM dithiothreitol (DTT), 0.02% NaN_3, and 50 μg/ml phenylmethylsulfonyl fluoride. The low ionic strength extract was adjusted to 1/4 strength of the column equilibration buffer before being concentrated by ultrafiltration. Concentrated samples were adjusted to full strength of column buffer prior to gelfiltration. Purified spectrin dimer was again concentrated by ultrafiltration to 2–3 mg/ml protein and dialyzed against buffer A [50 mM Tris–HCl, 10 mM KCl, 40 mM NaCl (pH 7.4)].

2.2. Phosphorylation of spectrin dimer

Phosphorylation was assayed in buffer A by incubating spectrin dimer at 0.05–1.8 mg/ml for 90 min, if not otherwise indicated, at 30°C in a total volume of 50 or 100 μl containing 5 mM $MgCl_2$, and [γ-^{32}P]ATP (PB 19168, Radiochemi-

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cal Center, Amersham) at the given concentration and specific activity. Cyclic AMP-dependent protein kinase from bovine heart (P 5511, Sigma, St. Louis) with the capacity to transfer 40–90 pmol phosphate per min at pH 6.5 and 30°C and 5 μM cAMP (A 4137, Sigma) were added as indicated. Alternatively, catalytic subunit of the cAMP-dependent protein kinase from bovine heart (P 2645, Sigma) was dissolved in 50 mM DTT and an amount was added that had a similar phosphorylating capacity to that of the whole enzyme. Reactions were initiated by the addition of label and stopped with electrophoresis sample buffer containing SDS, EDTA and DTT. Samples were denatured for 3 min at 100°C and the reduced material alkylated with excess *N*-ethylmaleimide.

2.3. *Polyacrylamide gel electrophoresis and one-dimensional peptide analysis*

Alkylated samples were electrophoresed on a Neville type SDS–PAGE [14] as in [15]. The running gel contained 4.5% polyacrylamide. The gels were stained and dried as in [16]. Incorporation of label into spectrin was revealed by autoradiography using Kodak X-Omat SO 282 films. Dried gels were exposed to films in intensifier cassettes (Kodak) at −70°C. Incorporation of label into spectrin was quantitated by Cerenkov emission from gel pieces containing both spectrin bands from either unstained or stained gels. Peptide maps from proteins were obtained essentially as in [17]. Briefly, proteins were electrophoresed on 4.5% SDS–PAGE and the spectrin region was identified on unstained gels from refractive changes at the edges of the two spectrin bands or from parallel gel lanes that were quickly stained. Gel pieces containing both spectrin bands were cut and marked to identify the direction of electrophoretic migration. These pieces were equilibrated in stacking gel buffer [17]. Excess fluid was removed from them by touching the pieces on cellophane. Several pieces (up to 9) were arranged on a new glass plate with spectrin band 1 being to the left of spectrin band 2 in each case. Running gel (15% acrylamide) and stacking gel were cast with the gel pieces in the proper place. To establish electric contact and to obtain an even, horizontal upper edge, the gel was first mounted into the electrophoresis chamber and 1% agarose in stacking gel buffer was introduced around the gel pieces. After solidification, electrophoresis buffer was added and 8–12 μg V_8 protease from *Staphylococcus aureus* (Miles, Milan) was underlaid in 0.5 ml stacking gel buffer containing 20% glycerol. Electrophoresis was carried out for 2 h at 15 mA, for 2 h at 6 mA and for 4–5 h at 20 mA.

2.4. *Spectrin and ankyrin from [^{32}P]phosphate-labeled red cells*

To compare the p*I* values of spectrin and ankyrin with that of a cAMP-dependent phosphoform of spectrin dimer, spectrin and ankyrin were obtained from [^{32}P]phosphate-labeled red cells as follows: red cells freed from white cells were washed 3 times in 145 mM NaCl, 5 mM KCl, 10 mM imidazole, 0.5 g/l D-glucose and 0.5 mM EDTA (pH 7.4) and once in the buffer that was later used for incubation, except that it lacked phosphate. Red cells were [^{32}P]phosphate labeled at 28% hematocrit overnight as in [18]. The buffer given contained 45 mM NaCl and 5 mM KCl instead of 50 mM NaCl and was further supplemented with 200 units per ml penicillin G, 50 μg/ml phenylmethylsulfonyl fluoride, 0.14–0.2 mCi/ml of carrier-free [^{32}P]-phosphate and 50–100 μM unlabeled phosphate. Labeled cells were washed 3 times in phosphate-buffered saline. Membranes were isolated and spectrin extracts obtained as outlined. IOV were isolated from spectrin-extracted membranes and washed once in extraction buffer and once in hemolysis buffer. Resuspended IOV and spectrin extracts were supplemented with 1% SDS and 5 mM *N*-ethylmaleimide and frozen in small samples. Ankyrin is enriched in IOV and is the predominant ^{32}P-labeled high molecular mass component in IOV. Hence IOV served as a source for ankyrin.

2.5. *Two-dimensional separation of polypeptides*

Spectrin extracts and IOV (enriched in ankyrin) from [^{32}P]phosphate-labeled red cells and in vitro labeled spectrin dimer were subjected to isoelectric focusing (IEF) as in [19]. The samples were applied to the anodic side and the electrode solutions were 10-times less concentrated than given there. After IEF the polypeptides were separated in the second dimension on SDS–PAGE. Autoradiographs from stained and dried gels are shown.

3. RESULTS

Purified spectrin dimer shows autophosphorylation [2–4]. Autophosphorylation was strongly dependent on the ATP concentration (fig.1,2). Spectrin phosphorylation was enhanced by addition of a cAMP-dependent protein kinase (cAMP-PK) at all ATP concentrations studied (fig.1). The cAMP-dependent phosphorylation of spectrin resulted in labeling of both spectrin bands with spectrin band 2 being predominantly labeled (fig.1). The labeling of spectrin band 1 was, however, higher when a reducing agent was present during incubation and was more pronounced than in fig.1, when the concentration of spectrin was kept low during incubation (cf. fig.1 and fig.2 inset). Addition of a cAMP-PK stimulated ^{32}P incorporation into spectrin 2.8-fold at 1 mM ATP, and 6.5- and 7.5-fold at 0.1 and 0.01 mM [γ-^{32}P]ATP respectively (table 1). Enhanced phosphorylation required cAMP and a cAMP-PK and was not observed with either cAMP or cAMP-PK alone. The labeling of spectrin occurred via phosphoryl transfer, because ATP [γ-^{35}S] could be substituted for [γ-^{32}P] ATP (see fig.5). Hence purified spectrin dimer can be phosphorylated in vitro by a heterologous cAMP-PK.

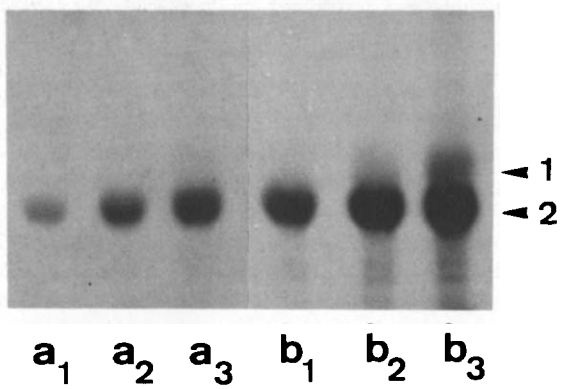

Fig.1. Autophosphorylation and cAMP-dependent phosphorylation of spectrin dimer. Autophosphorylation and cAMP-dependent phosphorylation of spectrin dimer (1.8 mg/ml) were carried out at 10_1, 100_2, and $1000\,\mu M$ [γ-^{32}P]ATP (123 mCi/mmol in all cases). Denatured samples containing $26\,\mu g$ spectrin dimer were electrophoresed on each lane. An autoradiograph of the spectrin region is shown. 1 and 2, spectrin band 1 and band 2. (a) Autophosphorylation, (b) phosphorylation in the presence of a cAMP-PK.

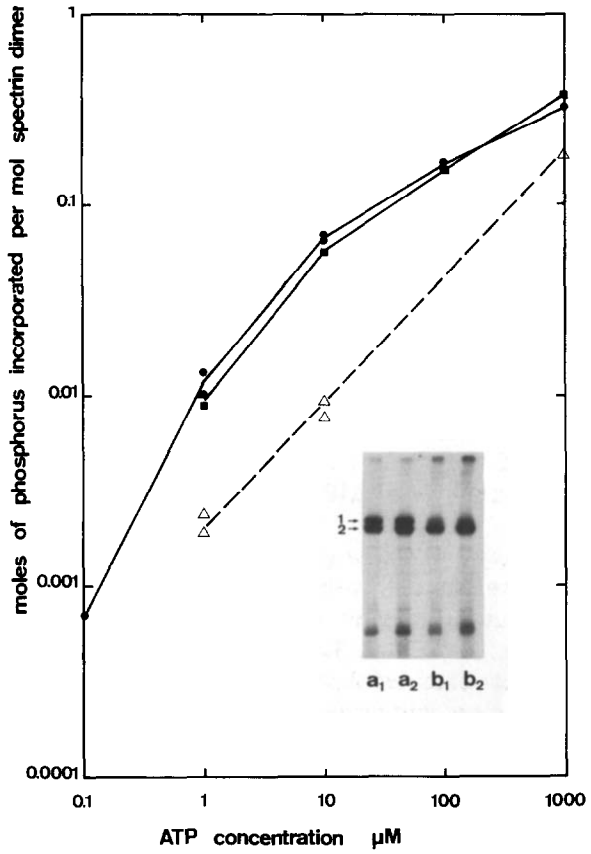

Fig.2. Extent of phosphorylation. Spectrin dimer (0.4 mg/ml) was phosphorylated in the presence or absence of cAMP-PK as described. Equal amounts of [γ-^{32}P]ATP ($6\,\mu Ci$) but increasing total ATP concentrations were used to quantitate incorporation of label. Samples were incubated in the presence of 1 mM dithiothreitol (■) or in the absence of reducing agent (●). The latter condition was achieved by alkylating mercaptoethanol which was provided with [γ-^{32}P]ATP with equimolar amounts of *N*-ethylmaleimide. Incorporation of label was determined as outlined. Total labeling was not significantly different whether analyzed in the presence or absence of reducing agent. Qualitatively, however, reducing conditions favored appearance of label in spectrin band 1 as is evident from the inset that shows the labeling pattern obtained by incubating spectrin (0.216 mg/ml) for 1 h (1) and 2 h (2) with cAMP-PK under reducing (a) and unreducing conditions (b). (●,■) Phosphorylation of spectrin dimer in the presence of a cAMP-PK, (△) autophosphorylation of spectrin dimer.

The cAMP-dependent phosphorylation of spectrin reached similar extents with or without reducing agent (fig.2). It reached at best 0.46 mol per

Table 1

Phosphorylation of spectrin dimer in the presence of a cAMP-dependent protein kinase

Additions	[γ-^{32}P]ATP (mM)	Extent of stimulation	Incorporation of phosphorus (mol/mol spectrin)
-	1	1	(n = 5) range 0.03–0.182
cAMP (5 μM)	1	1	(n = 2)
Protein kinase	1	1.4	(n = 3)
Protein kinase + cAMP	1	2.8	(n = 7) range 0.064–0.46
	0.1	5.5	(n = 3) range 0.051–0.08
	0.01	7.5	(n = 3) range 0.06–0.077
Catalytic subunit	1	6.8	(n = 2)

mol spectrin (fig.2) and 0.6 mol per mol with catalytic subunit of cAMP-PK. The extent of modification was, however, variable (table 1). This was due to several reasons: since the reaction required 90 min to reach the observed extent of incorporation, minute activities of phosphodiesterase could degrade cAMP and thus yield a lower stimulation than that found with catalytic subunit alone. This was not the only difficulty, however, because even the extent of autophosphorylation was variable. The cause of the latter variability is not known. While these results strongly suggest a cAMP-dependent phosphorylation of spectrin, phosphopeptide maps in fact demonstrate differences between autophosphorylated and a cAMP-dependent phosphoform of spectrin.

Phosphorylated spectrin was studied on one-dimensional peptide maps as in [17]. This type of peptide map allows comparison of the peptides generated from both spectrin subunits separately of several samples on the same gel (fig.3). The cAMP-dependent phosphoform of spectrin revealed label in several peptides of spectrin band 2 and in a few distinct peptides of band 1 (fig.3c,d). The entire phosphopeptide pattern was similar whether generated from spectrin labeled at 10 μM [γ-^{32}P]ATP at high specific activity (fig.3b) or at 1 mM [γ-^{32}P]ATP and low specific activity (fig.3c,d). It was, however, different from the simple pattern observed with autophosphorylated spectrin dimer at any ATP concentration (fig.3a). Thus, the cAMP-dependent phosphoform of spectrin band 1 contained 2–3 phosphopeptides and that of spectrin band 2 several, two of which were also labeled in autophosphorylated spectrin band 2, while the majority was distinct from autophos-

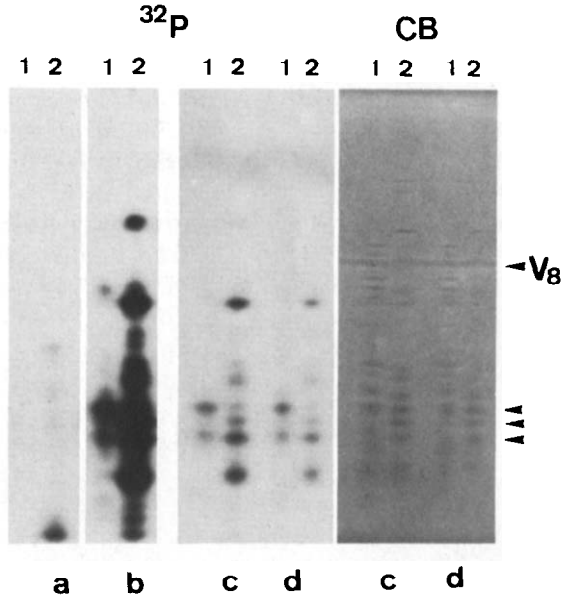

Fig.3. Peptide and phosphopeptide maps of in vitro phosphorylated spectrin dimer. Purified spectrin dimer was incubated in the absence (a) and presence of a cAMP-PK (b–d) at 10 μM [γ-^{32}P]ATP (12 900 cpm/pmol) in a and b and at 1 mM [γ-^{32}P]ATP (68 cpm/pmol) in c and d. The concentration of spectrin dimer was varied from 0.05 to 0.4 mg/ml. After incubation equal volumes of the denatured samples were subjected to peptide analysis. The data shown were obtained at 0.1 (c) and 0.05 mg/ml of spectrin dimer (d). Essentially the same phosphopeptide maps were obtained up to 0.4 mg/ml, except that phosphorylation of spectrin band 1 did not significantly increase with increasing concentrations of spectrin dimer. An autoradiograph (^{32}P) and a Coomassie blue-stained gel (CB) are shown. The arrows on the right point to phosphopeptides with similar apparent molecular masses in both spectrin bands. V_8 indicates the position of V_8 protease on SDS–PAGE. The numbers (1/2) on top indicate the position of the spectrin bands.

phorylated ones. The newly labeled peptides did not originate from phosphorylated protein of the added enzyme, because gel regions equivalent to those subjected to peptide analysis, but from a lane loaded with enzyme alone, did not contain labeled peptides (not shown). In fact, the similarity of the labeling pattern at high and low extent of modification and the coincidence of labeled phosphopeptides with well resolved, stained peptides of spectrin indicate that peptides of spectrin rather than of an impurity were phosphorylated.

The 3 cAMP-dependent phosphopeptides of spectrin band 1 had similar molecular masses to three phosphopeptides originating from band 2. The labeling of the phosphopeptides of spectrin band 1 did not increase with increasing concentrations of spectrin, while that of spectrin band 2 derived peptides did (fig.3c,d). Thus, spectrin band 2 was predominantly labeled at high spectrin concentrations.

Autophosphorylated spectrin, ankyrin, and the cAMP-dependent phosphoform of spectrin dimer were also analyzed by IEF and subsequent SDS–PAGE (fig.4). Autophosphorylated spectrin band 2 and ankyrin were positioned exactly as reported for the system applied [19]. The cAMP-dependent phosphoform of spectrin band 2 revealed the majority of label and protein at a p*I* slightly shifted towards a more alkaline pH as compared to that of autophosphorylated spectrin band 2 and contained some label comigrating with ankyrin. The cAMP-dependent phosphoform of spectrin band 2 was apparently highly sensitive to breakdown. Breakdown products were always detected underneath this, but not under the material that still migrated with the p*I* of autophosphorylated spectrin band 2. These results clearly indicate that a cAMP-dependent phosphorylation of spectrin generated new species that were different from phosphorylation spectrin band 2.

In an attempt to obtain some insight into the mechanism of a cAMP-dependent phosphorylation of spectrin, the effects of phosphatases and of 2,3-diphosphoglycerate (2,3-DPG) were analyzed. 2,3-DPG is known to suppress the cAMP-independent phosphorylation and autophosphorylation of

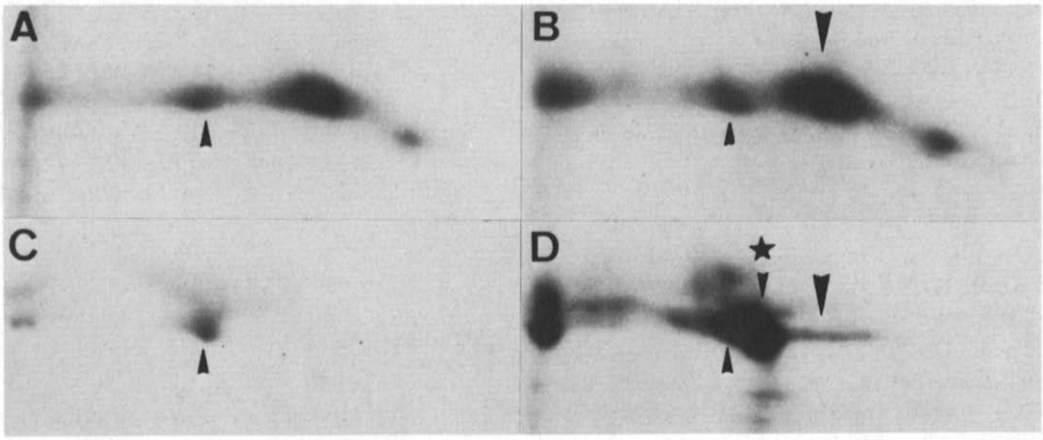

Fig.4. Two-dimensional separation of phosphorylated spectrin and ankyrin. Spectrin dimer was phosphorylated in the presence or absence of a cAMP-PK with 1 mM [γ^{32}P]ATP as given in fig.1. These phosphoforms of spectrin were compared to those of ankyrin from IOV and of spectrin both from [^{32}P]phosphate-labeled red cells as outlined in section 2. Similar amounts of autophosphorylated and cAMP-dependent phosphoform of spectrin dimer (28 μg) and IOV containing 100 μg protein were subjected to isoelectric focusing from left to right. Gel pieces were then electrophoresed in the second dimension on SDS–PAGE from top to bottom. Autoradiographs from the gel portions containing the spectrin/ankyrin region are shown. The heavy arrow points to the position of ankyrin. The small arrow from below the spot points to the position of autophosphorylated spectrin band 2 and the small arrow with asterisk indicates the position of the cAMP-dependent phosphoform of spectrin band 2. (A,B) ^{32}P-labeled high molecular mass proteins of IOV from red cells that were [^{32}P]phosphate labeled in the absence and presence of 10 μM cAMP, respectively. Cyclic AMP stimulates phosphorylation of ankyrin [20], (C) autophosphorylated spectrin dimer, (D) cAMP-dependent phosphoform of spectrin dimer.

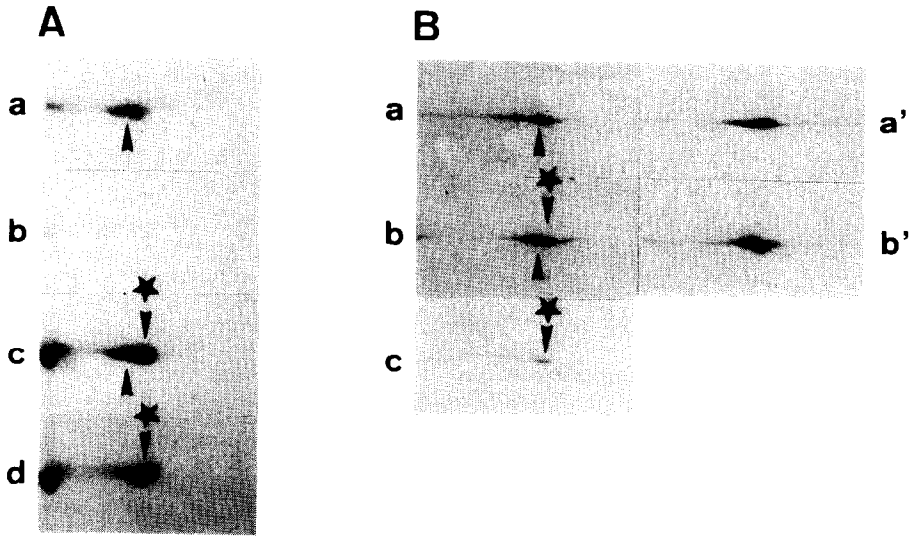

Fig.5. Effects of 2,3-DPG and of alkaline phosphatase on spectrin phosphorylation. Spectrin dimer (1.05 mg/ml in A and 1.26 mg/ml in B) was phosphorylated for 2 h in the presence or absence of cAMP and a cAMP-PK as described except for the following changes. In A [γ-^{32}P]ATP was added at 100 μM containing 8.3 μCi in a total volume of 50 μl; in B phosphorylation was assayed with 10 μM ATP[γ-^{35}S] containing 5 μCi in the same volume. Adenosine-5'-[γ-[^{35}S]thio]triphosphate was from New England Nuclear and the unlabeled material from Boehringer, Mannheim. Alkaline phosphatase (Boehringer, Mannheim) was added where indicated at 1 unit/ml and 2,3-DPG (Boehringer) at 10 mM. After incubation equal volumes of denatured samples were analyzed by two-dimensional separation as described in fig.4 and section 2. Arrows as in fig.4. (A) Phosphorylation of spectrin dimer with [γ-^{32}P]ATP was carried out with the following additions: a, none; b, 2,3-DPG; c, cAMP and cAMP-PK; d, cAMP, cAMP-PK and 2,3-DPG. (B) Phosphorylation of spectrin dimer with ATP[γ-^{35}S] was carried out with the following additions: a, none; b, cAMP and cAMP-PK; c, cAMP, cAMP-PK, and 2,3-DPG; a' and b' as in a and b, but with alkaline phosphatase.

spectrin [21]. This was confirmed for autophosphorylation as shown by autoradiographs from two-dimensional gels (fig.5A,a,b). Likewise, 2,3-DPG lowered but did not eliminate incorporation of label into spectrin, when phosphorylation was studied with a cAMP-PK (fig.5Ac,d). The differential susceptibility of the two phosphoforms towards 2,3-DPG allowed visualtization of the cAMP-dependent phosphate incorporation independently of phosphopeptide analyses. It further suggested that 10 mM 2,3-DPG could have suppressed autophosphorylation by preventing dephosphorylation of native spectrin, because 2,3-DPG could itself be a substrate for a contaminating or spectrin-bound phosphatase activity. In clarifying this point native spectrin dimer was dephosphorylated by insoluble alkaline phosphatase (Sigma). While a gentle treatment slightly stimulated phosphate incorporation with or without cAMP-PK, extensive dephosphorylation always resulted in breakdown products primarily of spectrin band 2. This breakdown was not inhibited by PMSF. The availability of the phosphatase-resistant ATP[γ-^{35}S] (Amersham), however, allowed a simultaneous dephosphorylation of native spectrin and ^{35}S–phosporyltransfer. In this assay system phosphorylation of spectrin was similarly enhanced by addition of phosphatase whether analyzed in the presence or absence of a cAMP-PK (fig.5B) or its catalytic subunit (not shown). Hence, the stimulatory effect of phosphatase was primarily on autophosphorylation rather than additive, e.g., on both types of phosphorylation. The cAMP-dependent phosphorylation of spectrin was, however, smaller with ATP-[γ-^{35}S] (fig.5B) than with [γ-^{32}P]ATP (fig.5A), as is evident from the label incorporated in the presence of 2,3-DPG. The cause of this difference remains to be investigated. Nevertheless, both lines of evidence are in agreement and suggest that dephosphorylation of native spectrin is required for autophosphorylation (as has been suggested earlier [22]), but not for the cAMP-dependent phosphorylation of spectrin.

4. DISCUSSION

The data shown demonstrate for the first time a cAMP-dependent phosphorylation of spectrin dimer with label in both bands. Spectrin band 2 contained in addition to autophosphorylated peptides several phosphopeptides that were distinct from autophosphorylated ones. While phosphorylation of spectrin band 2 is due to both autophosphorylation and a cAMP-dependent phosphorylation, that of spectrin band 1 is exclusively cAMP-dependent. It is conceivable that the cAMP-dependent phosphorylation of spectrin band 1 requires reducing conditions, but there is no explanation for why it decreases relative to that of spectrin band 2 with increasing concentrations of spectrin. Authors in [4] carried out similar experiments with exogenous cAMP-PK from a different source, but used cyanogen bromide fragmentation of the dimer instead of both bands separately. They suggested the incorporation label to reside primarily in spectrin band 1 and considered this a non-specific reaction, because it was observed with isolated spectrin. This conclusion is clearly not correct, because spectrin undergoes a cAMP-dependent phosphorylation in both bands, not only in vitro as shown here, but also in ghosts [5–7] and cytoskeletons [7], as well as in intact cells (unpublished). A cAMP-dependent phosphorylation of spectrin dimer was generally considered unlikely, because spectrin from [^{32}P]phosphate-labeled cells was exclusively phosphorylated in spectrin band 2 by autophosphorylation or cAMP-independent protein kinases [1,2]. This reasoning appears correct, but neglects the above-mentioned facts as well as the possibility that a cAMP-dependent phosphorylation of spectrin dimer, if it occurs in vivo, may alter the binding properties of the modified spectrin. Such a modification could result in ionic binding to the membrane and prevent a cAMP-dependent phosphoform of spectrin from extraction by low ionic strength buffers.

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