CK2 constitutively associates with and phosphorylates chicken erythroid ankyrin and regulates its ability to bind to spectrin

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Summary

Previous analyses have shown that the phosphorylation state of chicken erythroid ankyrin regulates its association with the spectrin cytoskeleton in vivo. Treatment of erythroid cells with serine and threonine phosphatase inhibitors stimulates the hyperphosphorylation of ankyrin and its dissociation from spectrin. In this study, we demonstrate that a kinase that directs the phosphorylation of ankyrin in vivo coprecipitates with ankyrin-containing complexes and has properties identical to CK2. Studies using CK2-specific inhibitors have indicated that all of the phosphorylation events associated with erythroid ankyrin in vivo are CK2 dependent. Furthermore, inhibitor studies combined with in vitro binding analyses have indicated that the phosphorylation of erythroid ankyrin by CK2 regulates its ability to associate with spectrin. Additional analyses revealed that CK2 coprecipitates with ankyrin-3-containing complexes isolated from Madin Darby canine kidney epithelial cells and phosphorylates this epithelial ankyrin isoform in vivo. These results are the first demonstration of a kinase constitutively associating with the ankyrin-spectrin cytoskeleton in erythroid and kidney epithelial cells. This association provides a mechanism for rapidly reorganizing the membrane cytoskeleton in these cell types through the phosphorylation of ankyrin.

Key words: Cytoskeleton, Ankyrin, Regulation, Erythroid, Epithelial

Introduction

The gene products encoded by the members of the ankyrin gene family are localized in multiple intracellular membrane compartments. At these various sites, ankyrin polypeptides function as linkers between diverse membrane proteins and the spectrin-based membrane cytoskeleton. The interactions mediated by ankyrin family members have been implicated in the sorting and stabilization of membrane proteins (Nelson and Hammerton, 1989; De Matteis and Morrow, 2000; Bennett and Chen, 2001) and are critical for the maintenance of plasma membrane stability and cell shape (Tse and Lux, 1999). Other investigators have shown that the cytoskeleton-binding properties of some components of the membrane cytoskeleton, including dematin (Husain-Chishti et al., 1989), adducin (Fukata et al., 1999) and protein 4.1 (Subrahmanyam et al., 1991), are regulated by phosphorylation. These phosphorylation-dependent changes in cytoskeleton binding result in dramatic changes in cell shape (Fukata et al., 1999) and stability (Khanna et al., 2002). Although the phosphorylation state of the membrane proteins neurofascin (Tuvia et al., 1997), the β1 subunit of the sodium channel (Malholtra et al., 2002), and $CD44v_{3,8\text{-}10}$ (Bourguignon et al., 1999) regulates their association with ankyrin, little is known regarding the mechanisms that regulate the dynamic association of ankyrin with other membrane protein receptors or spectrin in vivo.

Our recent studies have shown that the spectrin-binding properties of chicken erythroid ankyrin are regulated by phosphorylation (Ghosh and Cox, 2001). Treatment of chicken embryonic erythroid cells with serine and threonine phosphatase inhibitors stimulated the hyperphosphorylation of the 225 kDa and 205 kDa erythroid ankyrin isoforms. Ankyrin hyperphosphorylation correlated with a reduced association of ankyrin-AE1 complexes with cytoskeletal spectrin. In vitro binding studies have shown that the dissociation of ankyrin-AE1 complexes from the spectrin cytoskeleton is at least partially due to the reduced ability of hyperphosphorylated ankyrin to bind to spectrin. Thus, ankyrin phosphorylation represents a critical mechanism for regulating the cytoskeletal association of ankyrin-bound membrane proteins.

The studies described here have shown that a kinase activity that mediates the in vivo phosphorylation of ankyrin is constitutively associated with ankyrin-containing complexes isolated from chicken embryonic erythroid cells. Immunological and biochemical assays have shown that this associated kinase has properties identical to protein kinase CK2. Studies using CK2-specific inhibitors have suggested that all of the phosphorylation events associated with both basally phosphorylated and hyperphosphorylated ankyrin in vivo are dependent upon CK2. Furthermore, binding studies have indicated that the CK2-dependent phosphorylation of erythroid ankyrin regulates its ability to associate with spectrin in vitro. Additional analyses revealed that CK2 is constitutively associated with ankyrin 3 (ank3)-containing complexes isolated from Madin Darby canine kidney (MDCK) epithelial cells, and it phosphorylates this epithelial ankyrin isoform in vivo. The association of CK2 with ankyrin-

containing complexes in erythroid and epithelial cells provides a mechanism for rapidly altering the organization of the membrane cytoskeleton in these cell types through the CK2-dependent phosphorylation of ankyrin.

Materials and Methods

Immunoprecipitations

Erythroid cells from 10-day-old chicken embryos were lysed in isotonic buffer containing 1% Triton X-100 and separated into detergent-soluble and -insoluble fractions (Ghosh et al., 1999). Alternatively, whole cell lysates were prepared by directly lysing cells in immunoprecipitation buffer (Ghosh et al., 1999). Protein A-agarose beads preloaded with ankyrin-specific antibodies (Ghosh et al., 1999) were added to the various fractions and incubated overnight at 4°C. Following washing in immunoprecipitation buffer, immune complexes were released by incubation in SDS sample buffer and analyzed on a 6% SDS polyacrylamide gel. Immunoprecipitates were also prepared from a whole cell lysate from MDCK cells using a rabbit antibody that recognizes ank3 (Doctor et al., 1998), the major epithelial ankyrin isoform.

^{32}P-orthophosphate labeling of chicken erythroid cells

Erythroid cells from 10-day-old chicken embryos or MDCK cells were incubated in DMEM containing 1 mCi/ml ^{32}P-orthophosphate at 37°C for various times in the presence or absence of 100 nm calyculin A. Some of the cells were treated with 4,5,6,7-tetrabromobenzotriazole (TBB), a CK2-specific inhibitor (Sarno et al., 2001; Battistuta et al., 2001), during the labeling period. After labeling, the cells were detergent-fractionated, and ankyrin immunoprecipitates were prepared and analyzed on a 6% SDS polyacrylamide gel. The gels were stained with GelCode Blue (Pierce) and dried. ^{32}P-labeled species were detected by autoradiography.

In vitro kinase assays

Immunoprecipitates prepared as described above were washed three times with kinase buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM EGTA, 6 mM β-mercaptoethanol). The precipitates were then resuspended in 100 μl of kinase buffer containing 10 μCi of [γ-^{32}P]-ATP or [γ-^{32}P]-GTP and incubated at 37°C for 30 minutes. The reactions were terminated by the addition of SDS sample buffer. The samples were analyzed on a 6% SDS polyacrylamide gel, and ^{32}P-labeled species were detected by autoradiography. In some instances, 5 mM EDTA, 50 μg of heparin, 10 μM emodin (Battistuta et al., 2000) or 10 μM TBB was added during the in vitro kinase reaction. Alternatively, immunoprecipitates were boiled for 10 minutes, which eliminated the activity of the coprecipitating kinase. These heat-treated precipitates were incubated with 15 mU of purified CK2 from rat liver (Sigma) for 30 minutes at 37°C and analyzed as described above.

In gel kinase assays

A whole cell lysate was prepared from erythroid cells isolated from 10-day-old chicken embryos. This lysate and an ankyrin immunoprecipitate prepared from the lysate were electrophoresed on a 12.5% SDS polyacrylamide gel containing either 50 μg/ml myelin basic protein or 50 μg/ml of chicken erythroid membranes (Cox et al., 1985). After electrophoresis, the gels were washed with 20% isopropanol in 50 mM Tris-HCl pH 8.0 followed by two washes in 50 mM Tris-HCl pH 7.5 and 5 mM β-mercaptoethanol. Proteins in the gel were denatured by incubating the gel in 6 M guanidinium-HCl for 1 hour. The denaturation buffer was discarded and the proteins were allowed to renature in 50 mM Tris-HCl, 5 mM β-mercaptoethanol and 0.05% Tween-20 overnight at 4°C. After two washes in kinase buffer, the gels were incubated in kinase buffer containing 25 μM ATP and 100 μCi [γ-^{32}P] ATP for 45 minutes at 37°C. The gels were then washed extensively with 5% TCA containing 1% sodium pyrophosphate, dried and exposed to Biomax MS X-ray film.

Immunoblotting analysis

Immunoprecipitates were prepared from a whole cell lysate from 10-day-old embryonic erythroid cells using ankyrin preimmune and immune antisera. The precipitates were electrophoresed on a 12.5% SDS polyacrylamide gel and transferred to nitrocellulose. The filter was incubated with a 1:1,000 dilution of a rabbit polyclonal antibody that recognizes the α and α′ catalytic subunits of CK2 (Kikkawa et al., 1992). The filter was then washed and incubated with protein A conjugated to horseradish peroxidase. Following washing, immunoreactive species were detected by enhanced chemiluminescence. Immunoblotting analyses were used to determine the proteins in MDCK cells that are recognized by the ank3-specific antiserum (Doctor et al., 1998). A whole cell lysate from MDCK cells was electrophoresed on a 6% SDS polyacrylamide gel and processed for immunoblotting using a 1:2,000 dilution of the ank3-specific serum.

One-dimensional phosphopeptide mapping

Ankyrin immunoprecipitates were prepared from the detergent-soluble and -insoluble fractions of control or calyculin-A-treated erythroid cells. Some of the precipitates were prepared from cells labeled with ^{32}P-orthophosphate as described above. The remaining precipitates were prepared from unlabeled cells. Unlabeled precipitates were incubated in 100 μl of kinase buffer containing 10 μCi [γ-^{32}P]-ATP or heat-treated prior to incubation with purified CK2 from rat liver in kinase buffer containing [γ-^{32}P]-ATP. In each instance, the precipitates were resolved on a 6% SDS polyacrylamide gel. Following GelCode Blue staining, individual ankyrin isoforms were excised from the gel and electroeluted in a buffer composed of 125 mM Tris-HCl pH 6.8, 0.1% SDS, 1.0 mM EDTA and 30 mM DTT. The eluted proteins were digested with 10 ng of *Staphylococcus aureus* strain V8 endoproteinase at 37°C for 30 minutes. The resulting phosphopeptides were analyzed on 18% SDS polyacrylamide gels. The gels were dried, and ^{32}P-labeled peptides were detected by autoradiography.

Ank3 immunoprecipitates prepared from unlabeled MDCK cells were incubated in kinase buffer containing [γ-^{32}P]-ATP or heat-treated prior to incubation with purified CK2 from rat liver (Promega) in kinase buffer containing [γ-^{32}P]-ATP. The precipitates were electrophoresed on a 6% SDS polyacrylamide gel, and individual ank3 isoforms were isolated and digested with V8 protease as described above.

In vitro binding assay

An immunoprecipitate was prepared with an α-spectrin monoclonal antibody (ICN) from the detergent-insoluble fraction of erythroid cells that were lysed in isotonic buffer containing 1% Triton X-100. This precipitate was washed into a low salt buffer (10 mM NaCl, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 2 mM EGTA, 6 mM β-mercaptoethanol, and 1% Triton X-100). The precipitate was then incubated overnight at 4°C with ankyrin that was immunopurified from control erythroid cells or from erythroid cells that were incubated with calyculin A, calyculin A plus TBB or TBB alone as described below. Following extensive washing in low salt buffer, the α-spectrin immunoprecipitates were processed for immunoblotting analysis using a 1:2,000 dilution of the ankyrin-specific antiserum or a 1:1,000 dilution of α-spectrin-specific monoclonal antibody.

Control erythroid cells, or cells that were incubated in the presence

of 100 nM calyculin A, 100 nM calyculin A plus 60 μM TBB or 60 μM TBB for 2 hours were lysed in isotonic buffer containing 1% Triton X-100. These cells were separated into soluble and insoluble fractions by centrifugation, and ankyrin antibodies directly conjugated to cyanogen-bromide-activated Sepharose 4B beads were used to immunoprecipitate ankyrin from the detergent insoluble fraction. Following washing in isotonic buffer containing 1% Triton X-100, immunoprecipitated ankyrin was eluted from the beads in 0.2 M glycine, pH 2.3. The eluted ankyrin was dialyzed against low salt buffer and quantified by immunoblotting analysis. An equivalent amount of ankyrin from each sample was incubated with an α-spectrin immunoprecipitate in a total volume of 250 microliters.

Quantitative densitometry

Coomassie-stained gels were scanned using DeskScan II 2.2 software and quantitative densitometry was performed using NIH Image. The incorporation of ^{32}P into the erythroid and epithelial ankyrin isoforms was quantified by phosphoimager analysis using Image-Quant software (Molecular Dynamics).

Results

A kinase activity that phosphorylates chicken erythroid ankyrin is constitutively associated with ankyrin containing complexes

Previous studies have shown that detergent-insoluble ankyrin in chicken embryonic erythroid cells exists in spectrin-dependent and spectrin-independent pools (Ghosh and Cox, 2001). Treatment of cells with serine and threonine phosphatase inhibitors, which stimulate ankyrin hyperphosphorylation, dissociated both the spectrin-dependent and spectrin-independent pools of ankyrin from the detergent-insoluble cytoskeleton. Furthermore, in vitro binding studies have shown that the dissociation of ankyrin from cytoskeletal spectrin in cells treated with phosphatase inhibitors can be attributed to a reduced ability of hyperphosphorylated ankyrin to bind spectrin (Ghosh and Cox, 2001). Additional analyses have investigated whether the kinase activity that mediates the in vivo phosphorylation of ankyrin is constitutively associated with the erythroid ankyrin isoforms. Detergent-soluble and -insoluble ankyrin immunoprecipitates prepared from chicken embryonic erythroid cells were incubated in kinase buffer containing [γ-^{32}P]-ATP and subsequently analyzed by SDS gel electrophoresis and autoradiography. This experiment revealed that a kinase activity coprecipitates with both the detergent-soluble and -insoluble forms of ankyrin. This in vitro activity phosphorylated the insoluble 225 kDa and 205 kDa isoforms, and the soluble 225 kDa isoform from control cells (Fig. 1, lanes 5 and 6). Furthermore, the detergent-insoluble 220 kDa ankyrin isoform, which is not phosphorylated in vivo (Fig. 1, lane 2), was phosphorylated in vitro (Fig. 1, lane 6). Similar results were obtained with immunoprecipitates prepared from cells treated with the serine and threonine phosphatase inhibitor, calyculin A. The hyperphosphorylated 225 kDa and 205 kDa ankyrin isoforms from calyculin-A-treated cells, which are primarily detergent-soluble, were phosphorylated in vitro by this coprecipitating kinase activity (Fig. 1, lanes 7 and 8). In addition, longer exposure of the autoradiogram revealed that the detergent-insoluble 220 kDa isoform from calyculin-A-treated cells could serve as a substrate for this kinase in vitro (Fig. 1, lane 8*).

Characterization of a kinase activity that coprecipitates with ankyrin using an in gel kinase assay

To characterize the kinase activity that coprecipitates with ankyrin, in gel kinase assays were performed. The substrate used for these assays was a crude membrane preparation isolated from chicken erythroid cells. Analysis of a lysate from 10-day-old embryonic erythroid cells using this in gel assay revealed polypeptides of ~140 kDa, ~100 kDa and ~45 kDa with kinase activity (Fig. 2, lane 2). Although the ~140 kDa and ~100 kDa polypeptides could also phosphorylate the promiscuous substrate myelin basic protein, the ~45 kDa kinase only utilized erythroid membranes as a substrate (Fig. 2, lanes 1 and 2). In addition, none of these proteins were detected when substrate was omitted from the gel matrix (data not shown). A similar analysis with an ankyrin immunoprecipitate prepared from a whole cell lysate from 10-day-old embryonic red cells detected a single polypeptide of ~45 kDa that could utilize the erythroid membrane preparation as a substrate for phosphorylation (Fig. 2, lane 3).

The immunological and biochemical properties of the kinase that coprecipitates with ankyrin are similar to CK2

Purified CK2 from human red blood cells phosphorylates human erythroid ankyrin in vitro (Wei and Tao, 1993). Interestingly, the ~45 kDa polypeptide in ankyrin immunoprecipitates that was detected in the in gel kinase assay is similar in size to the predicted molecular weight of the α catalytic subunit (M_r 45,190) of chicken CK2 (Maridor et al.,

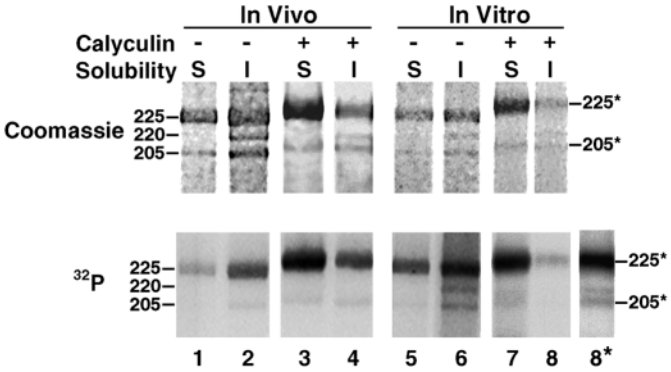

Fig. 1. Phosphorylation of chicken erythroid ankyrin in vivo and in vitro. Ankyrin immunoprecipitates were prepared from the detergent-soluble (S) and -insoluble (I) fractions from erythroid cells labeled with ^{32}P-orthophosphate for 4 hours (lanes 1-4) or from unlabeled cells (lanes 5-8). Some of the cells used for these analyses were incubated in the presence of 100 nM calyculin A for 4 hours prior to detergent lysis (lanes 3, 4, 7, and 8). Immunoprecipitates from unlabeled cells were incubated in kinase buffer containing [γ-^{32}P]-ATP for 30 minutes at 37°C prior to gel analysis (lanes 5-8). Immune complexes were resolved on a 6% SDS polyacrylamide gel. Individual ankyrin isoforms were visualized by staining with GelCode Blue, and ^{32}P-labeled species were detected by autoradiography. Lane 8* is a five-fold longer exposure of lane 8. The migrations of the 225 kDa, 220 kDa and 205 kDa ankyrin isoforms are indicated to the left of the figure. The migration of the hyperphosphorylated 225 kDa (225*) and 205 kDa (205*) isoforms are indicated to the right of the figure.

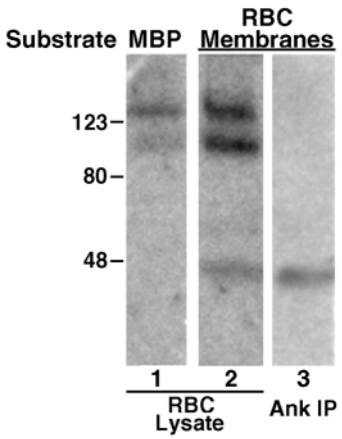

Fig. 2. Characterization of the erythroid ankyrin-associated kinase using an in gel kinase assay. A whole cell lysate was prepared from erythroid cells isolated from 10-day-old chicken embryos. This lysate (lanes 1 and 2) and an ankyrin immunoprecipitate prepared from the lysate (lane 3) were electrophoresed on a 12.5% SDS polyacrylamide gel containing either 50 μg/ml myelin basic protein (lane 1) or 50 μg/ml of chicken red blood cell membranes (lanes 2 and 3). Following electrophoresis, the gels were processed as described in the Materials and Methods and incubated in kinase buffer containing 25 μM ATP and 100 μCi [γ-^{32}P] ATP. The gel was then washed and dried, and ^{32}P-labeled species were detected by autoradiography. Molecular weight markers are indicated to the left of the figure.

1991). To investigate the relationship between the coprecipitating kinase and CK2, an ankyrin immunoprecipitate prepared from a whole cell lysate from embryonic erythroid cells was processed for immunoblotting using antibodies that recognize the α and α′ catalytic subunits of CK2 (Kikkawa et al., 1992). This analysis revealed that the CK2 antibody detected a polypeptide of ~45 kDa in the ankyrin immunoprecipitate (Fig. 3, lane 3) that was not detected in the precipitate prepared with preimmune sera (Fig. 3, lane 2). This suggests that the α or α′ CK2 subunit, or a related CK2 isoform, is associated with ankyrin-containing complexes in these cells.

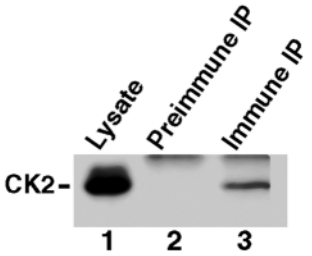

Fig. 3. CK2 coprecipitates with chicken erythroid ankyrin. Whole cell lysate prepared from erythroid cells from 10-day-old chicken embryos (lane 1) or immunoprecipitates prepared from this lysate using ankyrin preimmune (lane 2) or immune sera (lane 3) were electrophoresed on a 12.5% SDS polyacrylamide gel and transferred to nitrocellulose. This filter was incubated with a rabbit antibody that recognizes the α and α′ catalytic subunits of CK2, followed by protein A conjugated to horseradish peroxidase. After washing, immunoreactive species were detected by enhanced chemiluminescence.

Having demonstrated that a CK2-like protein coprecipitates with erythroid ankyrin, we investigated whether the kinase that phosphorylates ankyrin in vitro possessed biochemical properties similar to CK2. The catalytic activity of CK2 is Mg^{2+}-dependent, and the addition of EDTA to the in vitro kinase reaction completely inhibited ankyrin phosphorylation (Fig. 4A, lane 2). The activity of CK2 is also heparin-sensitive, and the inclusion of heparin in our in vitro kinase assays almost entirely blocked the ability of the coprecipitating kinase to phosphorylate ankyrin (Fig. 4A, lane 3). Another hallmark of CK2 is its ability to use GTP as well as ATP as a phosphate

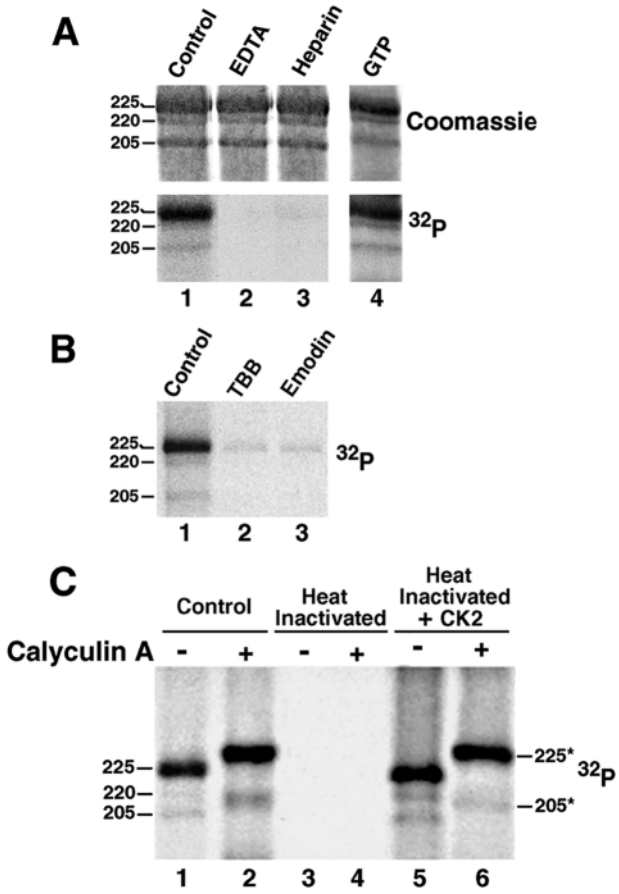

Fig. 4. Biochemical properties of the ankyrin-associated kinase. Ankyrin immunoprecipitates prepared from a whole cell lysate from erythroid cells were incubated in kinase buffer containing [γ-^{32}P]-ATP (A and B, lanes 1-3) or [γ-^{32}P]-GTP (A, lane 4) for 30 minutes at 37°C. CK2 inhibitors, including 5 mM EDTA (A, lane 2), 50 μg of heparin (A, lane 3), 10 μM TBB (B, lane 2) and 10 μM emodin (B, lane 3), were added to some of the immunoprecipitates during the in vitro kinase reaction. Alternatively, ankyrin precipitates prepared from whole cell lysates from control (C, lanes 1, 3, 5) or calyculin-A-treated erythroid cells (C, lane 2, 4, 6) were either incubated in kinase buffer containing [γ-^{32}P]-ATP for 30 minutes at 37°C (C, lanes 1 and 2) or boiled for 2 minutes before the kinase reaction (C, lanes 3 and 4). To some of the heat-treated precipitates, purified CK2 from rat liver was added prior to incubation at 37°C for 30 minutes (C, lanes 5 and 6). Following the kinase reactions, samples were analyzed on 6% SDS polyacrylamide gels. The migration of the 225 kDa, 220 kDa, and 205 kDa ankyrin isoforms are indicated by dashes to the left of each panel. Dashes to the right of the panel C indicate the hyperphosphorylated (225*) 225 kDa and (205*) 205 kDa ankyrin isoforms.

donor during the phosphorylation reaction. The kinase that coprecipitates with ankyrin efficiently utilized GTP as a phosphate donor during in vitro assays (Fig. 4A, lane 4).

We also examined the effect of the CK2-specific inhibitor TBB on the activity of the coprecipitating kinase. Until recently, highly specific inhibitors for CK2 have not been available. However, other investigators have shown that 10 μM TBB inhibits 87% of the in vitro activity of rat liver CK2 while having little or no effect on 32 other kinases that were assayed (Sarno et al., 2001). As shown in Fig. 4B (lane 2), 10 μM TBB significantly blocked the activity of the coprecipitating kinase. Quantification of multiple experiments has revealed that TBB inhibits 90.2±1.2% (n=2) of the coprecipitating kinase activity. A similar result was observed with emodin (Fig. 4B, lane 3), another CK2 inhibitor (Battistuta et al., 2000). Taken together, these data indicate that CK2 is constitutively associated with erythroid ankyrin-containing complexes and mediates the phosphorylation of ankyrin in our in vitro assays.

To directly determine whether chicken erythroid ankyrin can serve as a substrate for CK2-dependent phosphorylation, ankyrin immunoprecipitates prepared from whole cell lysates from control or calyculin A treated cells were boiled prior to the vitro kinase reaction. This treatment completely eliminated the activity of the coprecipitating kinase (Fig. 4C, lanes 3 and 4). These heat-inactivated precipitates were used as a substrate for purified CK2 isolated from rat liver. This analysis revealed that rat liver CK2 could phosphorylate each of the ankyrin isoforms from control cells in vitro (Fig. 4C, lane 5). In addition, hyperphosphorylated ankyrin from calyculin-A-treated cells was efficiently utilized as a substrate by purified CK2 (Fig. 4C, lane 6).

CK2 is the major ankyrin kinase in chicken erythroid cells

One-dimensional peptide mapping was used to compare the phosphorylation sites in ankyrin immunoprecipitated from control and calyculin-A-treated erythroid cells. This analysis yielded identical phosphopeptide maps for the detergent-soluble and -insoluble 225 kDa ankyrin isoforms precipitated from cells labeled with ^{32}P-orthophosphate in the presence of calyculin A (Fig. 5, lanes 3 and 4). Some of the phosphorylated peptides from hyperphosphorylated ankyrin were held in common with the phosphopeptides generated from the detergent-insoluble 225 kDa isoform precipitated from ^{32}P-orthophosphate-labeled control cells (Fig. 5, lane 5). However, several phosphopeptides were uniquely associated with ankyrin precipitated from control or calyculin-A-treated cells. These distinct patterns of phosphorylation probably contribute to the differing abilities of basally phosphorylated and hyperphosphorylated ankyrin to associate with spectrin (Ghosh and Cox, 2001).

The profile of phosphopeptides observed for the in vitro phosphorylated 225 kDa isoform (Fig. 5, lane 6) was similar to the phosphopeptide map of the in vivo phosphorylated 225 kDa isoform precipitated from control cells (Fig. 5, lane 5; comigrating peptides are marked with asterisks). This result suggests that the kinase activity that coprecipitates with ankyrin can mediate most of the ankyrin phosphorylation events that occur in control cells in vivo. In addition, the in vitro phosphorylated 225 kDa (Fig. 5, lane 6) and 220 kDa

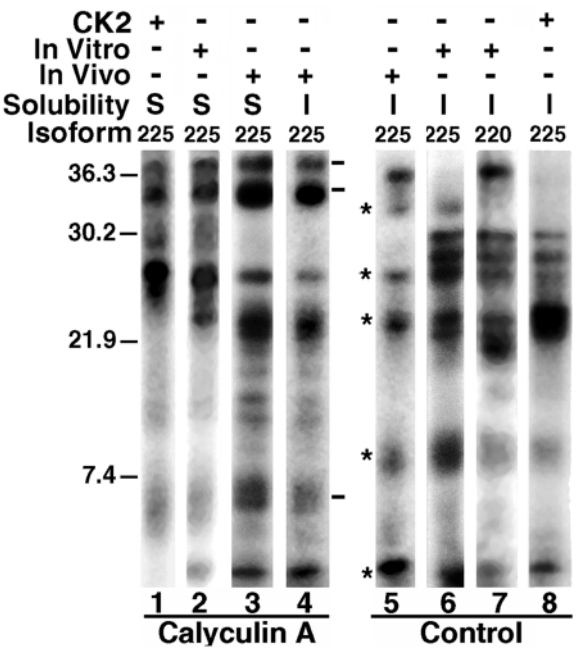

Fig. 5. One-dimensional phosphopeptide mapping analysis of erythroid ankyrin isoforms. Ankyrin immunoprecipitates were prepared from the detergent soluble (lanes 1-3) and insoluble fractions (lanes 4-8) of control (lanes 5-8) or calyculin A treated erythroid cells (lanes 1-4). Some of the precipitates were prepared from cells labeled with ^{32}P-orthophosphate (lanes 3-5). The remaining precipitates, which were prepared from unlabeled cells, were phosphorylated in vitro by the coprecipitating kinase (lanes 2, 6 and 7) or by purified CK2 (lanes 1 and 8). In each instance, the precipitates were resolved on a 6% SDS polyacrylamide gel, and the gel was stained with GelCode Blue. The indicated ankyrin isoforms were excised from the gel, electroeluted, and the eluted proteins were digested with V8 endoproteinase. The resulting phosphopeptides were analyzed on 18% SDS polyacrylamide gels. The gels were dried, and ^{32}P-labeled peptides were detected by autoradiography. Molecular weight markers are indicated to the left of the figure.

(Fig. 5, lane 7) isoforms precipitated from control cells shared similar phosphopeptide maps. This indicates that the lack of phosphorylation of the 220 kDa isoform in vivo is not simply due to the fact that this isoform lacks the sequences that are phosphorylated in the 225 kDa isoform in vivo. The phosphopeptide map of the in vitro phosphorylated 225 kDa ankyrin isoform from control cells (Fig. 5, lane 6) lacked all of the phosphopeptides uniquely associated with ^{32}P-labeled ankyrin precipitated from calyculin-A-treated cells (Fig. 5, lanes 3 and 4). However, many of these unique phosphopeptides (marked with dashes in Fig. 5) were generated when ankyrin precipitated from calyculin-A-treated cells was used as a substrate for in vitro phosphorylation (Fig. 5, lane 2).

To investigate whether purified CK2 phosphorylates a similar array of sites on ankyrin as the coprecipitating kinase, we compared the one-dimensional phosphopeptide maps of the 225 kDa ankyrin isoform that had been phosphorylated by these kinases. This analysis revealed very similar phosphopeptide maps for the 225 kDa ankyrin isoform from control cells that was phosphorylated by the coprecipitating

kinase (Fig. 5, lane 6) or by purified CK2 (Fig. 5, lane 8). Interestingly, purified CK2 also phosphorylated many of the phosphopeptides uniquely associated with the hyperphosphorylated 225 kDa ankyrin isoform (Fig. 5, lanes 3 and 4) when ankyrin from calyculin-A-treated cells was used as a substrate (Fig. 5, lane 1).

The data described above strongly suggest that the majority of the phosphorylation events associated with both basally phosphorylated and hyperphosphorylated erythroid ankyrin in vivo are mediated by CK2. Furthermore, the in vivo phosphorylation sites in the 225 kDa ankyrin isoform that are not observed in vitro may be caused by bound antibody blocking the phosphorylation of these sequences in vitro. To further investigate the role of CK2 in directing ankyrin phosphorylation, we examined the effect of the CK2-specific inhibitor, TBB, on ankyrin phosphorylation in vivo. This analysis revealed that TBB completely blocked the in vivo phosphorylation of ankyrin in control and calyculin-A-treated erythroid cells (Fig. 6), suggesting that the phosphorylation of ankyrin in chicken erythroid cells is entirely CK2 dependent. This reagent also prevented the shift of ankyrin from the detergent-insoluble to the soluble pool that results when erythroid cells are treated with calyculin A (Fig. 6, compare the Coomassie-stained profiles in lanes 5 and 6 with those in lanes 7 and 8).

The ability of TBB to block the calyculin-A-induced changes in the solubility of ankyrin suggested a critical role for CK2 in regulating the cytoskeleton-binding properties of erythroid ankyrin. To address this possibility, binding studies have examined whether the CK2-dependent phosphorylation of ankyrin directly affects its capacity to associate with spectrin in vitro. For these analyses, erythroid cells were lysed in isotonic buffer containing 1% Triton X-100, and α-spectrin immunoprecipitates were prepared from the detergent-insoluble fraction in the same buffer. The precipitates were washed into low salt buffer and incubated with ankyrin that was immunopurified from control erythroid cells or from cells that were treated with calyculin A, calyculin A plus TBB or TBB alone as described in the Materials and Methods. Following washing of the α-spectrin immunoprecipitates in low salt buffer, they were subjected to immunoblotting analysis with ankyrin-specific or α-spectrin-specific antibodies (Fig. 7). As shown previously, chicken erythroid ankyrin dissociates from spectrin when cells are lysed in isotonic buffer containing 1% Triton X-100 (Fig. 7, lane 1). When ankyrin from control cells was incubated with the α-spectrin in low salt buffer, the 225 kDa ankyrin isoform coprecipitated with spectrin (Fig. 7, lane 4). Longer exposure of the ankyrin immunoblot revealed that the 205 kDa ankyrin isoform from control cells also bound to spectrin (data not shown). In contrast, there was no detectable binding of hyperphosphorylated ankyrin that had been immunopurified from calyculin-A-treated cells to spectrin (Fig. 7, lane 2). However, ankyrin immunopurified from cells treated with calyculin A plus TBB (Fig. 7, lane 3) or TBB alone (Fig. 7, lane 5) bound to spectrin to a similar extent to ankyrin from control cells. These results, which were obtained in two independent experiments, indicate that the CK2-dependent hyperphosphorylation of ankyrin dramatically inhibits its ability to associate with spectrin in vitro. These data further suggest that CK2 is the major regulator of the cytoskeleton-binding properties of ankyrin in chicken embryonic erythroid cells.

CK2 phosphorylates ankyrin 3 isoforms in MDCK kidney epithelial cells

We also studied whether CK2 serves as a regulator of ankyrin

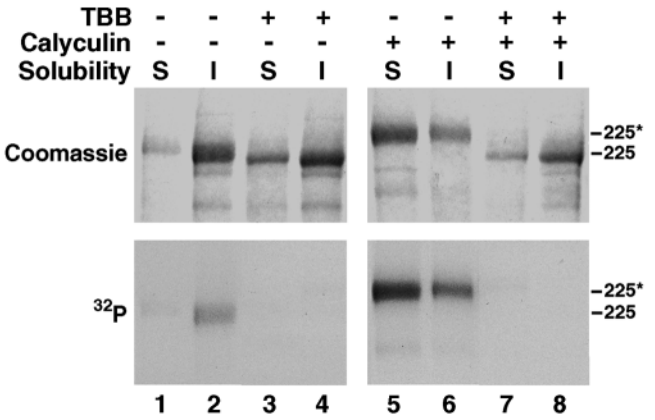

Fig. 6. TBB inhibits the basal phosphorylation and the hyperphosphorylation of chicken erythroid ankyrin in vivo. Erythroid cells from 10-day-old chicken embryos were incubated in DMEM containing 1 mCi/ml ^{32}P-orthophosphate at 37°C for 2 hours in the absence (lanes 1-4) or presence (lanes 5-8) of 100 nm calyculin A. Some of the cells were also treated with 60 μM TBB (lanes 3, 4, 7, and 8) during the labeling period. After labeling, the cells were detergent-fractionated and ankyrin immunoprecipitates were prepared and analyzed on a 6% SDS polyacrylamide gel. Immunoprecipitated polypeptides were visualized by staining with GelCode Blue, and ^{32}P-labeled species were detected by autoradiography. Dashes to the right of each panel indicate the basally phosphorylated (225) and hyperphosphorylated (225*) 225 kDa ankyrin isoform.

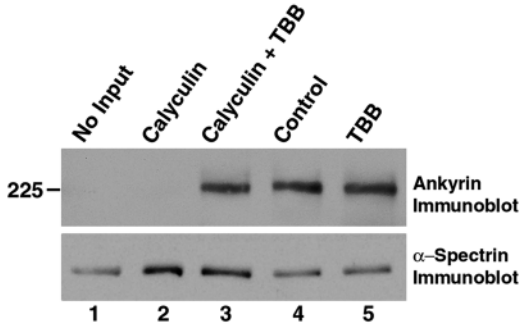

Fig. 7. CK2-dependent phosphorylation of ankyrin regulates its ability to associate with spectrin in vitro. Erythroid cells were lysed in isotonic buffer containing 1% Triton X-100, and an α-spectrin immunoprecipitate was prepared from the resulting detergent insoluble fraction and washed into low salt buffer. The immunoprecipitate was then incubated with low salt buffer (lane 1) or with ankyrin that had been immunopurified from control cells (lane 4), from cells treated with calyculin A (lane 2), from cells treated with calyculin A plus TBB (lane 3) or from cells treated with TBB alone (lane 5). Following extensive washing in low salt buffer, the immunoprecipitates were split in half and processed for immunoblotting with ankyrin-specific or α-spectrin-specific antibodies. The dash to the left of the ankyrin immunoblot indicates the basally phosphorylated 225 kDa ankyrin isoform.

function in cell types other than erythroid cells. For these experiments, we used antibodies that recognize the major epithelial ankyrin isoform, ank3. Immunoblotting analysis of a whole cell lysate from MDCK cells with these antibodies revealed two major ank3 isoforms of 215 kDa and 200 kDa (Fig. 8A, lane 1), as well as two minor species of 170 kDa and 120 kDa (data not shown). To assess whether these ank3 isoforms were phosphorylated in a CK2-dependent manner, ank3 immunoprecipitates were prepared from ^{32}P-orthophosphate-labeled MDCK cells incubated in the absence or presence of the CK2-specific inhibitor, TBB. Coomassie staining of these immunoprecipitates revealed a profile similar to the immunoblot, with two major species of 215 kDa and 200 kDa (Fig. 8A, lanes 2 and 3). Although both isoforms were phosphorylated in vivo, the extent of phosphorylation was significantly inhibited in cells treated with TBB (Fig. 8A, lanes 4 and 5). Quantification of multiple experiments identical to that shown in Fig. 8A indicated that TBB treatment of cells resulted in a 53.9±8.7% (n=2) decrease in the phosphorylation of the ank3 isoforms. The residual ankyrin phosphorylation observed in TBB-treated cells suggests that other kinases are involved in the phosphorylation of ank3 in this epithelial cell type.

In vitro kinase assays with ank3 immunoprecipitates examined whether the kinase(s) involved in ank3 phosphorylation in vivo physically associate with ankyrin-containing complexes in MDCK cells. This analysis revealed that a kinase activity coprecipitated with ank3 and phosphorylated both the 215 kDa and 200 kDa ank3 isoforms in vitro (Fig. 8B, lane 2). This coprecipitating kinase also phosphorylated a species of 210 kDa, which was not visible in the Coomassie staining pattern of the ank3 immunoprecipitate (Fig. 8B). Like the erythroid ankyrin kinase, this MDCK cell kinase was heparin sensitive (Fig. 8B, lane 3), and incubation of a heat-inactivated precipitate with purified CK2 resulted in a profile of phosphorylated species identical to that observed with the coprecipitating kinase (Fig. 8B, lane 5). Finally, one-dimensional phosphopeptide mapping revealed that purified CK2 and the coprecipitating kinase phosphorylated similar sites in the 215 kDa (Fig. 9) and 200 kDa (data not shown) ank3 isoforms from MDCK cells. These results strongly suggest that CK2 is constitutively associated with ank3-containing complexes in MDCK cells and is likely to be involved in regulating ankyrin function in this epithelial cell type.

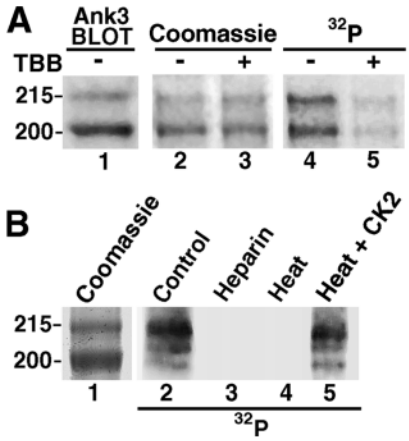

Fig. 8. In vivo and in vitro phosphorylation of ank3 isoforms from MDCK cells. A whole cell lysate from MDCK cells (A, lane 1) was resolved on a 6% SDS polyacrylamide gel, transferred to nitrocellulose, and processed for immunoblotting using rabbit antibodies specific for ank3. Ank3 antibodies were also used to prepare immunoprecipitates from MDCK cells labeled with ^{32}P-orthophosphate at 37°C for 4 hours in the absence (A, lanes 2 and 4) or presence (A, lanes 3 and 5) of 120 μm TBB. In addition, immunoprecipitates prepared from unlabeled MDCK cells were incubated in kinase buffer containing [γ-^{32}P]-ATP for 30 minutes at 37°C (B). In some instances, 50 μg of heparin was included during the in vitro kinase reaction (B, lane 3). Alternatively, the precipitate was boiled for 10 minutes, which eliminated the activity of the coprecipitating kinase (B, lane 4), and subsequently incubated with 15 mU of purified CK2 from rat liver (B, lane 5). Samples were analyzed on 6% SDS polyacrylamide gels. Immunoprecipitated polypeptides were visualized by staining with GelCode Blue, and ^{32}P-labeled species were detected by autoradiography. The migration of the 215 kDa and 200 kDa ank3 isoforms is indicated to the left of each panel.

Discussion

Previous studies have indicated that the phosphorylation state of ankyrin regulates its ability to associate with the detergent-insoluble cytoskeleton of chicken erythroid cells (Ghosh and Cox, 2001). Treatment of erythroid cells with serine and threonine phosphatase inhibitors stimulates the hyperphosphorylation of ankyrin and its release from the

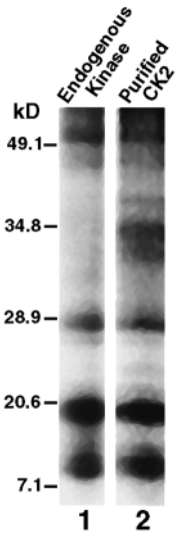

Fig. 9. One-dimensional phosphopeptide mapping of the 215 kDa ank3 isoform from MDCK cells. An ank3 immunoprecipitate prepared from MDCK cells was incubated in kinase buffer containing [γ-^{32}P]-ATP (lane 1), or was heat-treated prior to incubation with purified rat liver CK2 in kinase buffer containing [γ-^{32}P]-ATP (lane 2). Following the kinase reactions, the precipitates were analyzed on a 6% SDS polyacrylamide gel, and the gel was stained with GelCode Blue. The 215 kDa ank3 isoform from each in vitro reaction was excised from the gel, electroeluted, and the eluted protein was digested with V8 endoproteinase. The resulting phosphopeptides were analyzed on an 18% SDS polyacrylamide gel. The gel was dried, and ^{32}P-labeled peptides were detected by autoradiography. Molecular weight markers are indicated to the left of the figure.

spectrin cytoskeleton. The data presented here indicate that the phosphorylation events associated with both basally phosphorylated and hyperphosphorylated ankyrin in erythroid cells in vivo are dependent upon a kinase with properties identical to CK2. This kinase constitutively associates with ankyrin-containing complexes in embryonic erythroid cells and regulates the ability of ankyrin to associate with spectrin. The demonstration that CK2 also associates with ank3 isoforms from MDCK kidney epithelial cells suggests a widespread role for this kinase in regulating ankyrin function.

CK2 is a ubiquitous, highly conserved kinase involved in multiple cellular processes including cell division and proliferation (Li et al., 1999) and membrane protein trafficking (Cotlin et al., 1999; Mauxion et al., 1996; Shi et al., 2001). This multisubunit kinase is composed of α and α' catalytic subunits and a regulatory β subunit (Sarno et al., 2000), which associate to form $\alpha_2\beta_2$, $\alpha'_2\beta_2$, and $\alpha\alpha'\beta_2$ complexes that may have distinct functions in vivo (Dobrowolska et al., 1999). Although the subunit composition of the CK2 holoenzyme in chicken erythroid cells has not been defined, in gel kinase and immunoblotting analyses have shown that the size of the catalytic subunit of the ankyrin-associated kinase is very similar to the predicted size of the α catalytic subunit of chicken CK2 (Maridor et al., 1991).

The in vitro phosphorylation of ankyrin from control cells by coprecipitating CK2 did not yield the slower-migrating hyperphosphorylated form of ankyrin that is observed when erythroid cells are treated with phosphatase inhibitors in vivo. Furthermore, the phosphopeptides uniquely associated with hyperphosphorylated ankyrin from calyculin-A-treated cells could not be recapitulated when ankyrin from control cells was phosphorylated in vitro by coprecipitating CK2. These results must be reconciled with the inhibitor studies, which suggested that all of the phosphorylation events associated with both basally phosphorylated and hyperphosphorylated ankyrin in vivo are CK2 dependent. One explanation that could account for these results is that another CK2-dependent kinase is required to generate some of the unique phosphorylation events associated with hyperphosphorylated ankyrin in vivo. Alternatively, CK2 undergoes phosphorylation in calyculin-A-treated erythroid cells (data not shown). This modification may slightly alter the specificity of this kinase, leading to the phosphorylation events detected in hyperphosphorylated ankyrin.

The cytoskeletal 225 kDa ankyrin polypeptide from TBB-treated erythroid cells (Fig. 6, lane 4) migrated as a discrete species that comigrated with the lower half of the 225 kDa ankyrin species from untreated cells (Fig. 6, lane 2). This result suggests that cytoskeletal ankyrin in untreated cells exists in both a phosphorylated and a dephosphorylated state. Although the physiological consequences of the CK2-dependent phosphorylation events associated with basally phosphorylated ankyrin are not known, recent studies have shown that cytoskeletal ankyrin in chicken erythroid cells turns over with a relatively short half-life (Ghosh and Cox, 2001). Interestingly, CK2-dependent phosphorylation is involved in regulating the degradation of other ANK-repeat containing proteins, such as *Drosophila* Cactus (Liu et al., 1997) and its mammalian counterpart IκBα (Lin et al., 1996; McElhinny et al., 1996). Whether the basal phosphorylation events associated with a subset of cytoskeletal ankyrin polypeptides are involved in regulating their stability remains to be determined.

Unlike the other chicken erythroid ankyrin isoforms, the 220 kDa isoform is exclusively detergent insoluble, and treatment of cells with phosphatase inhibitors does not alter its solubility. This isoform is also unique in that it is not phosphorylated in vivo, although it can serve as a substrate for CK2 in vitro. The lack of phosphorylation of this isoform in vivo could be due to the fact that this protein is sequestered in a cellular compartment devoid of CK2, or alternatively the protein may assume an in vivo conformation that can not undergo phosphorylation. Further studies will be required to determine whether these or other mechanisms account for the observed in vivo properties of the 220 kDa isoform.

Crystal structure data have suggested that CK2 is constitutively active (Niefind et al., 2001). The fact that this kinase is also constitutively associated with ankyrin raises the question of how the level of ankyrin phosphorylation is regulated. The concentration of calyculin A used during our in vivo studies typically inhibits the multimeric serine and threonine phosphatases, PP1 and PP2A. Although it is not known whether the phosphorylation status of ankyrin in cells is directly regulated by PP1, PP2A or another cellular phosphatase, additional analyses have shown that in vitro phosphorylated ankyrin can be directly dephosphorylated by a phosphatase present in chicken erythroid cell lysates (data not shown).

Our previous studies demonstrated that chicken erythroid ankyrin-containing complexes can undergo dynamic rearrangements in response to changes in ankyrin phosphorylation. The results described here suggest that most, if not all of the phosphorylation events associated with erythroid ankyrin are mediated by CK2. Furthermore, we show that CK2 is constitutively associated with ankyrin in both erythroid and kidney epithelial cells. This is the first report of a kinase constitutively associating with elements of the membrane cytoskeleton in these cell types. Through this physical association, cells can rapidly regulate the interaction between ankyrin and spectrin and as a consequence alter the organization of their membrane cytoskeleton in response to extracellular signals.

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