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Structural domain mapping and phosphorylation of human erythrocyte pallidin (band 4.2)

Elizabeth Dotimas [a,1], David W. Speicher [c], Bipasha GuptaRoy [a,2] and Carl M. Cohen [a,b]

[a] *Dept. of Biomedical Research, St. Elizabeth's Hospital of Boston, Boston, MA (USA),* [b] *Dept. of Medicine and of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA (USA) and* [c] *The Wistar Institute, Philadelphia, PA (USA)*

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Pallidin (band 4.2) is a major protein of the human erythrocyte membrane, and plays an important but as yet undefined role in maintaining the normal shape and lifespan of the erythrocyte. The pallidin protein has been purified by a new procedure which yields a protein which is > 97% pure as judged by gel electrophoresis, while pallidin purified by our original procedure is only approx. 85% pure. The new form of the protein is unstable in physiological salt solutions. However, taking advantage of its high purity, we have used the new form of the protein to produce a structural domain map of its principal tryptic fragments. We also show that pallidin can be phosphorylated by a red-cell membrane kinase which partially co-purifies with it, and has properties similar to the catalytic subunit of cAMP-dependent kinase. Both cAMP-dependent kinase and the red-cell kinase phosphorylate the same tryptic domains on the pallidin protein. Our results show that endogenous pallidin on the red-cell membrane is a poor substrate for the kinase, possibly because it is fully phosphorylated, or inaccessible to the kinase.

Introduction

Pallidin is a peripheral red-cell membrane protein, tightly bound to the inner membrane surface and comprising approx. 5% of the total membrane protein [1]. Early studies of the red-cell membrane suggested that the membrane association of pallidin was via erythrocyte band 3, the anion transport protein [2], possibly in association with ankyrin [3,4]. Numerous clinical studies have shown that individuals whose red cells lack or are deficient in pallidin have abnormal or misshapen red cells and suffer from anemia, presumably due to accelerated red-cell destruction [5–10]. While such studies attest to the importance of pallidin for red-cell survival, the exact function of the protein has remained elusive.

We previously developed a procedure to extract and purify the protein from red-cell membranes and showed that pallidin bound saturably and with high affinity to the cytoplasmic domain of band 3 in red-cell inside-out vesicles stripped of endogenous pallidin [11,12]. In addition, we showed that pallidin formed a complex with ankyrin when both proteins were in solution, although we could find no evidence for an association between ankyrin and pallidin on the membrane. Even though ankyrin also binds to the cytoplasmic domain of band 3, we found no evidence for competition between ankyrin and pallidin for binding to alkaline-stripped red-cell inside-out vesicles [12].

Further information regarding pallidin has been provided by the cloning and sequencing of pallidin cDNA isolated from a human reticulocyte expression library [13,14]. The most intriguing finding to emerge from these studies is the discovery that the pallidin protein shares significant amino-acid sequence identity with the family of transglutaminase-type enzymes. Moreover, the structure of the gene for pallidin is remarkably similar to that of the genes for human coagulation Factor XIII subunit a, a secreted transglutaminase, and keratinocyte transglutaminase, an intracellular enzyme [15,16]. This similarity suggests that the gene for pallidin and the genes for the transglutaminases were derived from a common ancestral gene, and that pallidin and the transglutaminases share common structural or functional characteristics which have yet to be elucidated. Such characteristics could include recognition or binding sites for other proteins. More

Correspondence to: C.M. Cohen, Dept. of Biomedical Research, St. Elizabeth's Hospital of Boston, 736 Cambridge St., Boston, MA 02135, USA.

[1] Present address: Laboratory of Cell and Molecular Biology, New England Deaconess Hospital, 185 Pilgrim Rd., Boston, MA 02215, USA.

[2] Present address: Department of Biology, Brandeis University, Wattham, MA 02254, USA.

recently, non-erythroid pallidin has been implicated in the formation or function of intracellular vesicles including platelet dense granules, kidney lysosomes and melanosomes through its linkage to the murine *pallid* mutation [17]. This association has prompted the name pallidin for the band 4.2 protein.

The importance of pallidin to red-cell function, its close relationship to secreted and intracellular transglutaminases, and the widespread presence of pallidin analogues in non-erythroid cells [18] suggest that red-cell pallidin is a member of a class of proteins with varied cellular functions. Here we describe additional properties of pallidin which will likely be useful in the further characterization of both erythroid and non-erythroid pallidin proteins. We also show that the purified protein can be phosphorylated by a kinase which partially co-purifies with it, as well as by the catalytic subunit of cAMP-dependent kinase (PKA).

Materials and Methods

Histones, the regulatory and catalytic subunits of cAMP-dependent protein kinase (PKA; from bovine heart), PKI, heparin, alkaline phosphatase III-N, Kodak X-OMAT AR film, and TPCK-treated trypsin (Type XIII) were obtained from Sigma, St. Louis, MO. H9 was from Seikagaku America, Rockville, MD. Wiptide was from Peninsula Laboratories, Belmont, CA. [γ-^{32}P]ATP (7000 Ci/mmol) was from ICN Radiochemicals, Costa Mesa, CA. Immobilon-PVDF membrane was from Millipore, Bedford, MA.

Preparation of spectrin / actin / band 6-depleted vesicles for pallidin extraction. Preparation of ghosts and extraction of spectrin and actin followed published procedures [19–21] with some modifications. 600–700 ml of packed red cells were washed four times with 10 vols. of 150 mM NaCl, 5 mM sodium phosphate, 0.5 mM EGTA (pH 8.0). The packed cells were then resuspended in 1 volume of this buffer followed by incubation with 1.6 mM diisopropylfluorophosphate (final concentration) on ice for at least 1 h. These cells were lysed with 10 vols. of 5 mM sodium phosphate, 0.5 mM EGTA (pH 8.0) containing 20 μg/ml phenylmethylsulfonylfluoride (PMSF) and 2 μg/ml pepstatin A (lysis buffer) and immediately centrifuged for 20 min in a Sorvall GSA rotor at 12500 rpm. The resulting pelleted ghosts were washed four times with lysis buffer or until white.

Spectrin and actin were extracted in 15 vols. of 0.1 mM EGTA (pH 8.5) containing PMSF (20 μg/ml) and 0.2 mM dithiothreitol for 30 min at 37°C. The vesicles (referred to below as inside-out vesicles) were sedimented for 50 min in a Sorvall GSA rotor at 12500 rpm and then resuspended in lysis buffer to the same volume as the ghosts from which they were derived. Band 6 (glyceraldehyde-3-phosphate dehydrogenase) was removed by incubating the spectrin/actin-depleted inside-out vesicles with 10 vols. of 150 mM NaCl in lysis buffer for 20 min on ice, followed by centrifugation in a Sorvall GSA rotor at 12500 rpm for 45 min. The vesicles were washed once with lysis buffer and then resuspended in the same buffer to a third of the volume of the ghosts from which they were made. These membranes were used for the extraction and purification of pallidin as described below and in Results.

Extraction and purification of pallidin. The spectrin, actin, and band 6-depleted vesicles were mixed with an equal volume of 4 M Tris, 20 μg/ml PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin and 0.5 mM DTT (pH 7.2), and incubated for 30 min on ice with gentle stirring. The mixture was centrifuged in a Beckman 60Ti rotor at 35000 rpm for 35 min, and the supernatant was concentrated to < 50 ml in an Amicon ultrafiltration unit fitted with a YM30 filter. The concentrate was loaded onto a Sepharose 6B column (2.2 $\times$ 180 cm) and eluted with 2 M Tris-HCl, 0.5 mM EGTA, 0.02% NaN_3. 0.5 mM DTT (pH 7.2), at a flow rate of 20 ml/h. The fractions were monitored by A_{280} and examined by gel electrophoresis. Those fractions containing pallidin were pooled, concentrated to approx. 50 ml and dialyzed twice against 2 l of 5 mM sodium phosphate, 0.5 mM EGTA, 0.5 mM DTT (pH 7.2). After 12 h of dialysis, the contents of the bag (including any precipitate) was carefully removed, aliquoted into tubes, and centrifuged in an Eppendorf microfuge at 4°C for 10 min. The pallidin was recovered in the sedimented material. After removal of the supernatant, the pooled pellets were resuspended in a total final volume of 5.0 ml of 25 mM sodium phosphate (pH 11), 0.02% NaN_3, and 0.05% Tween 20. The resuspended material was dialyzed twice against 1 l of 5 mM sodium phosphate (pH 7.2), 0.5 mM EGTA, 0.5 mM DTT, followed by centrifugation as above for 10 min to remove aggregates. The final supernatant contains electrophoretically pure pallidin (see Results). Typically, the above procedure yields a total of about 2 mg of pallidin at a concentration of 400 μg/ml from 2 U of blood.

Limited proteolysis of pallidin by trypsin. Limited proteolysis of pallidin by TPCK-treated trypsin was carried out for 30 min on ice in 5 mM sodium phosphate, 0.5 mM EGTA (pH 8.0). Enzyme to substrate weight ratios were 1/250 or 1/50, and the digestion was terminated by adding 1 μl of a 1/50 dilution of diisopropylfluorophosphate in water, followed by Laemmli sample buffer. In some cases the digests were electrophoretically transferred to Immobilon membranes and subjected to N-terminal sequencing in a gas-phase sequenator.

Preparation of RBC kinase. RBC kinase was prepared by dialyzing the trailing edge of the pallidin peak

(pool b, Fig. 4B) obtained from the gel filtration of crude pallidin on Sepharose 6B (see above). Under these conditions all of the pallidin becomes insoluble and is removed by centrifugation at 12000 rpm for 10 min as noted above. This procedure leaves a clear supernatant which contains the bulk of the kinase activity associated with the original column fractions. (The sedimented pallidin (pool b) can be resuspended and contains a small amount of residual kinase activity. Pallidin pool a, treated in the same way contains no residual kinase activity.) The kinase was concentrated to a volume equal to the volume of resuspended pallidin, generally 5 ml, having a protein concentration of 80 μg/ml, and was used without further modification for the experiments described in the text. On heavily loaded gels the material contained bands of approx. 71, 55, and 35 kDa and no attempt was made to further purify the activity. The activity of the isolated kinase was stable for up to 3 months of storage on ice.

Kinase activity of 2 M Tris column fractions. 100 μl of every fourth fraction of the 2 M Tris Sepharose 6B column was assayed for kinase activity in 10 mM $MgCl_2$, 20 μM ATP, 10^6cpm [γ-^{32}P]ATP, 150 μg/ml histone in a total volume of 2.0 ml for 10 min at 30°C. The reaction was terminated by adding 0.5 ml of ice cold 50% TCA. 60 μl of 5% (w/v) BSA was added and the mixture was vortexed and left on ice for 10 min. The precipitate was filtered through 25-mm diameter Whatman GF/C filters, washed twice with 2.0 ml of ice cold 5% (w/v) TCA, and once with 95% ethanol, dried and counted for ^{32}P in a liquid scintillation counter. Samples were corrected for non-specific binding of ^{32}P to the filters by subtracting the ^{32}P counts of samples containing only of column elution buffer.

Phosphorylation of pallidin and inside-out vesicles. 10 μg of pallidin was phosphorylated in the absence or presence (see below) of added kinase in a medium containing 20 mM Tris-HCl (pH 7.5), 0.5 mM or 10 mM $MgCl_2$ (see figure legends), 20 μM ATP, 25×10^3 cpm/ml [γ-^{32}P]ATP at 30°C for 10 min or longer times as indicated in the text. The reaction was terminated either by the addition of 1 mM EDTA (final conc.) prior to use of ^{32}P-labeled pallidin for tryptic digestion, or by 5 × Laemmli sample buffer followed by boiling for 1 min and electrophoresing on SDS-polyacrylamide gels. In some experiments, 10 μg of pallidin was incubated with 5 μg of PKA catalytic subunit or 0.5 μg RBC kinase under the conditions described above. Quantitation of $^{32}PO_4$ incorporation into pallidin was done by two methods: 1. Following phosphorylation of pallidin in the presence of [γ-^{32}P]ATP and electrophoresis, pallidin bands of known mass were excised from an SDS-polyacrylamide gel, equilibrated overnight in scintillation fluid and counted in a scintillation counter. 2. Alternatively, $^{32}PO_4$ incorporation into pure pallidin was measured following TCA precipitation (in the presence of 100 μg bovine serum albumin as a carrier) onto Whatman GF/C filters as described in the previous section. Both methods gave similar results for pallidin phosphorylation stoichiometry.

Inside-out vesicles, pH 11 stripped inside-out vesicles, or pH 11 stripped inside-out vesicles reconstituted with pallidin (10–20 μg vesicles in 10 μl) were phosphorylated with 0.5 μg RBC kinase using the above conditions. Vesicles were subsequently solubilized in 5 × Laemmli sample buffer and electrophoresed as described below. Reconstitution of pallidin on membranes was done by incubating vesicles at a concentration of 0.2 mg/ml with 50 μg/ml pallidin in 20 mM KCl, 5 mM sodium phosphate (pH 7.2), 0.5 mM DTT, 20 μg/ml PMSF at 25°C for 4 h. Membranes were washed twice in 5 mM sodium phosphate (pH 7.2) prior to being resuspended and phosphorylated as described above.

Protein kinase assay for substrate specificity. RBC kinase (2 μg) or PKA (5 U) were assayed for protein kinase activity in a final volume of 80 μl containing 20 mM Tris-HCl (pH 7.6), 30 μM γ-^{32}P-ATP (500 cpm/pmol), 10 mM magnesium acetate, 10 mM sodium fluoride, 5 μg/ml leupeptin, 0.25 mM PMSF and 10 μg of substrate (see text). After incubation at 30°C for 10 min, 50 μl of the reaction mixture was spotted on a 2 × 2 cm square of phosphocellulose paper. The papers were washed twice for 10 min with stirring in 75 mM phosphoric acid, rinsed with 95% ethanol, dried and assayed for ^{32}P by scintillation counting.

Phospho-peptide mapping of pallidin. Purified pallidin phosphorylated in the presence of [γ-^{32}P]ATP by RBC kinase or PKA catalytic subunit as described above was digested with TPCK-treated trypsin at a weight to weight ratio of 1:250 for 30 min on ice in 5 mM sodium phosphate (pH 8.0), 0.5 mM EGTA. The reaction was terminated by adding boiling Laemmli sample buffer followed by boiling for 1 min and electrophoresis. The fragments were transferred onto Immobilon-P membrane for 18 h at 50 V in a buffer containing 12.5 mM Tris, 96 mM glycine, 10% methanol. The membrane was washed briefly in water (3 changes of water in 5 min) and stained with 0.025% Coomassie blue R250, 40% methanol for 5 min, destained in 50% methanol for 5 min and air-dried and autoradiographed on Kodak X-OMAT AR film with an intensifying screen. Coomassie blue stained bands containing ^{32}P activity were excised from the blot and subjected to N-terminal sequencing in a gas phase sequenator.

Alkaline phosphatase treatment of inside-out vesicles. 200 μl of inside-out vesicles were washed twice with at least 4 vols. of 20 mM Tris-glycine (pH 8.5) and resuspended in the same buffer. The vesicles were incubated with or without alkaline phosphatase (10 μg; 0.3 U) for 0–6 h in the presence of 5 μg/ml leupeptin, 2

μg/ml antipain, 2 μg/ml pepstatin A, and 20 μg/ml PMSF at 30°C and washed twice with 5 mM sodium phosphate, 0.5 mM EGTA (pH 7.2), 0.5 mM DTT. Subsequently, vesicles were phosphorylated as described above and subjected to SDS gel electrophoresis and autoradiography.

Electrophoresis. SDS gel electrophoresis [22,23] was carried out in 15% polyacrylamide gels for analysis of digested pallidin and 10% polyacrylamide gels for other samples. Gels were dried between dialysis membranes and autoradiographed using Kodak X-OMAT AR film and an intensifying screen.

Determination of protein concentration. Protein concentration of membranes was determined using the method of Lowry et al. [24] in the presence of 2% SDS and that of pallidin and the cytoplasmic domain of band 3 by the method of Bradford [25]. Bovine serum albumin was used as the standard in both methods.

Results

Fig. 1A shows a Coomassie-blue-stained SDS-gel of the membranes and extract used for the purification of pallidin (designated band 4.2 or 4.2 in the figures). Pallidin typically comprised 20% of the protein extracted by 2 M Tris (lane 4). Further purification of the crude pallidin preparation was achieved by chromatographing the mixture on a Sepharose 6B column (Fig. 1B,C). Fractions containing pallidin (labeled III, Fig. 1B) were pooled. In some cases, pool III was divided into two portions, a and b, with b containing little or no band 4.1 contamination. Pools I and II contained mainly ankyrin and band 4.1, respectively (Fig. 1C).

The fractions containing pallidin in Peak III were combined and analyzed by gel electrophoresis. Quantitative densitometry of the gel shown in Fig. 2, lane 1

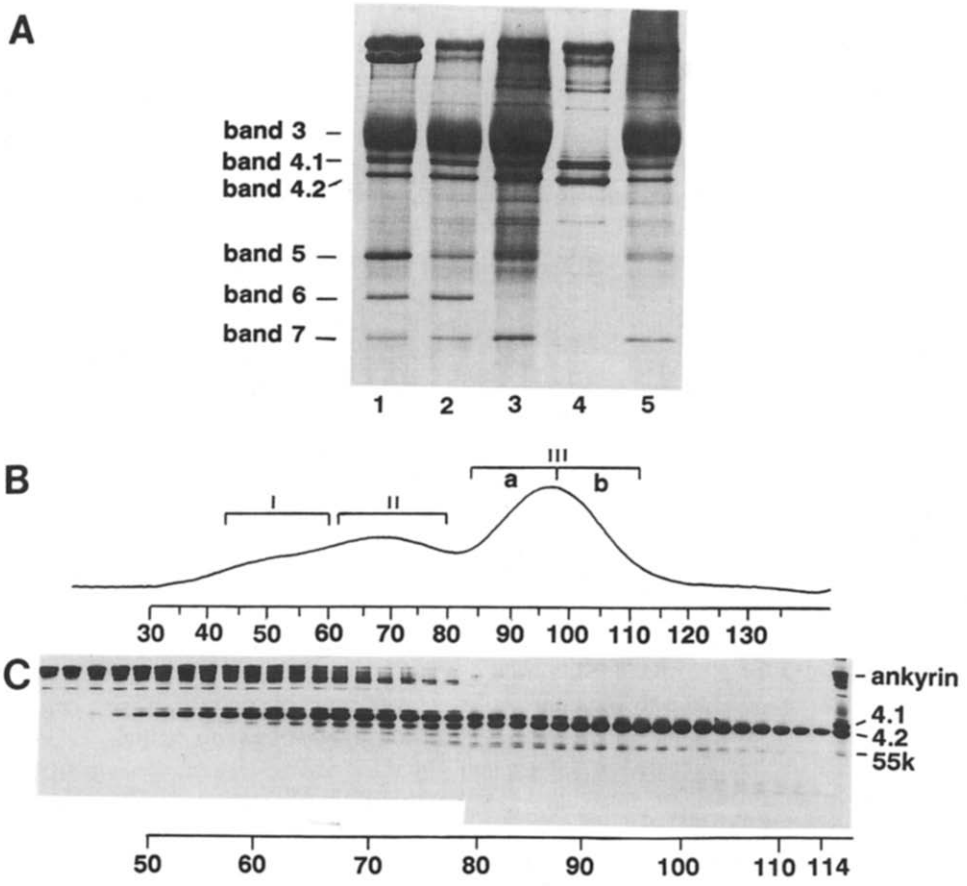

Fig. 1. (A) SDS-gel electrophoresis of red-cell membranes at various steps in the procedure used to purify pallidin (band 4.2). Lane 1: human erythrocyte ghosts; lane 2: ghosts depleted of spectrin and actin (IOVs: inside-out vesicles); lane 3: IOVs minus band 6; lane 4: 2 M Tris extract of membranes shown in lane 3; lane 5: 2 M Tris extracted membranes. (B) Elution profile of 2 M Tris extract on Sepharose 6B column. A_{280} profile of column elution. The column was run as described in Materials and Methods; the fractions were 3.0 ml each. Pool I contained principally ankyrin, pool II contained principally band 4.1 and pool III contained pallidin. (C) Fractions from the Sepharose 6B column electrophoresed on SDS-gels. 160 μl of each fraction was precipitated with trichloroacetic acid and electrophoresed as described in Materials and Methods.

revealed that 88% of the protein is pallidin, 5% is a 55-kDa protein and the remainder is smaller peptides. When this material was dialyzed (see Materials and Methods) to remove the 2 M Tris, the contents of the dialysis bag became turbid. Centrifugation of the turbid suspension yielded a supernatant consisting principally the 55-kDa protein, with traces of pallidin and an approx. 36-kDa protein (Fig. 2, lane 2). More than 99% of pallidin was found in the pellet (Fig. 2, lane 3). Quantitative densitometry indicated that this pelleted pallidin was $> 97\%$ pure, and contained $< 0.7\%$ contamination by the 55-kDa protein.

Attempts to prevent the precipitation of pallidin resulting from the dialysis of the 2 M Tris proved futile. Dialysis into increasing concentrations of NaCl, KCl or Tris (up to 1 M) had no effect. Likewise, dissolution of the precipitate in 6 M guanidine HCl followed by removal of the guanidine by dialysis did not prevent re-precipitation. It was found that a medium of high pH, 25 mM sodium phosphate, pH 11.0, containing 0.05% Tween 20 was successful at solubilizing the protein. When the pH-11-solubilized protein was brought to neutrality by dialysis against 5 mM sodium phosphate (pH 7.2), the contents of the dialysis bag remained clear and the pallidin stayed in solution. The resulting pallidin remained soluble in buffers of low to moderate ionic strength, e.g., 20–40 mM KCl but tended to aggregate in higher salt solutions. Because of this property the protein was not suitable for use in membrane binding studies.

The amino-acid composition of the purified pallidin protein was determined (not shown) and was found to be identical to the composition predicted from the pallidin cDNA sequence [13]. We subjected the purified pallidin protein to limited digestion with trypsin in order to generate a domain map of the protein (Fig. 3A). N-terminal sequencing of the major peptides was used to place the peptides (Fig. 3C) within the known amino-acid sequence of pallidin [13]. The bands labeled 40 kDa and 23 kDa were placed at the N-terminus of the protein by virtue of the fact that they both had blocked N-termini, as does intact pallidin [13]. Further confirmation of the placement of these bands is given below.

When purified pallidin was incubated with [γ-^{32}P]-ATP, the protein was found to incorporate ^{32}P covalently (Fig. 4A, lanes 1 and 4). To determine whether the kinase activity responsible for this phosphorylation was a co-purifying contaminant in the pallidin preparation, we analyzed the column fractions from the Sepharose 6B column from which the pallidin was eluted. Figs. 4B and 4C show that the column fractions contained several peaks of histone kinase activity, but that a major peak of kinase activity eluted with the trailing end of the pallidin profile. The purified pallidin shown in Fig. 4A was obtained from the column

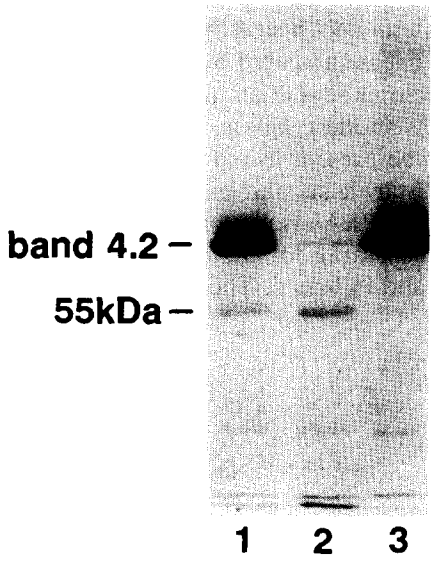

Fig. 2. Removal of low-molecular-weight contaminants from pallidin (band 4.2) column fractions. Lane 1: SDS gel of pooled pallidin-rich column fractions containing a 55-kDa and other lower-molecular-weight contaminants. In this preparation there was little or no band 4.1 contamination of the pallidin fractions. As described in Materials and Methods, these pooled fractions were concentrated and dialyzed. The resulting turbid solution was centrifuged at 12000 rpm for 10 min. Lane 2: the supernatant from that centrifugation, containing principally the 55-kDa protein and other lower-molecular-weight contaminants; lane 3: the pellet from that centrifugation, containing $> 97\%$ pure pallidin. This material was resuspended as described in Materials and Methods. Note the significant reduction in the 55-kDa protein in lane 3 relative to lane 1, computed by densitometry to be from 4.4% contamination to 0.7%.

fractions shown in Fig. 4B following the precipitation described above and we determined that the bulk of the kinase activity remained in the supernatant after precipitation of the pallidin. However, some residual kinase activity remained with the pallidin, largely in pool b.

The column fractions comprising the pallidin peak were divided into two pools corresponding to the leading (pallidin pool a) and trailing (pallidin pool b) halves and assayed for phosphorylation activity after precipitation and resuspension of the pallidin. Fig. 4A shows that pool a contained little or no detectable phosphorylating activity (lane 5) while pool b contained considerable phosphorylating activity (lane 6). The kinase activity remaining in the supernatant from the preparation of the pallidin pool b was saved (see Materials and Methods) and is referred to below as RBC kinase.

Further analysis of the RBC kinase activity present in the pallidin supernatants is shown in Fig. 3B. The RBC kinase, which has no detectable autophosphorylating activity (lane 1), was added back to pallidin pool a, which has no kinase activity of its own (lane 3). Fig. 3B, lane 4, shows that the kinase phosphorylated pallidin pool a. These results confirm that the kinase activity shown in Fig. 4 which co-chromatographs with the trailing end of the pallidin peak can be recovered

in the pallidin pool b supernatant, and that the pallidin pool a is uncontaminated by the kinase.

To determine whether pallidin could be phosphorylated by another kinase, we tested PKA which is known to be present in red cells. Fig. 3B lane 5 shows that the catalytic subunit of PKA phosphorylated purified pallidin. PKA also autophosphorylates as shown in lane 2, accounting for the presence of the additional labeled band in lane 5. The other minor bands in lane 5 are likely due to phosphorylation of minor contaminants or aggregates in the pallidin preparation.

We attempted to order the domain(s) phosphorylated by the RBC kinase and by PKA within the primary sequence of pallidin. Purified pallidin (pool a) was phosphorylated by each kinase and digested with trypsin. With the exception of the additional bands contributed by PKA autophosphorylation (Fig. 3B, lanes 8 and 11) the digestion pattern obtained using

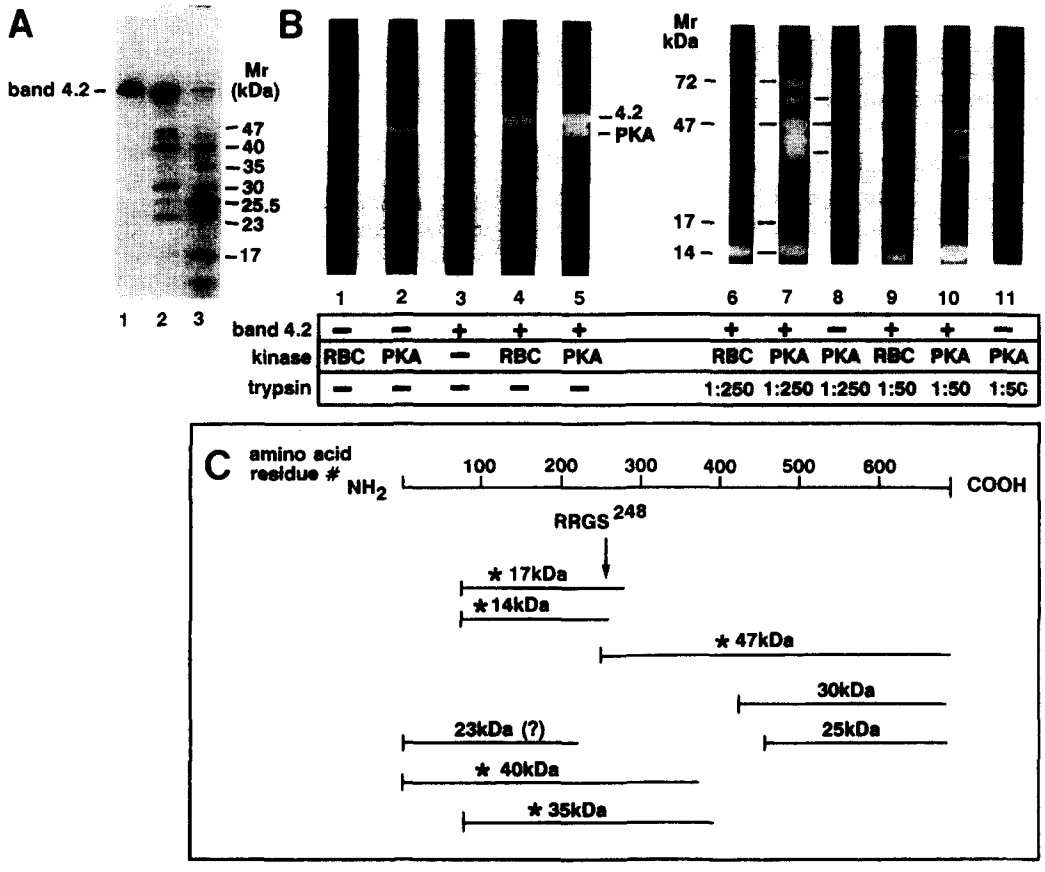

Fig. 3. Digestion and domain mapping of pallidin (band 4.2). (A) SDS gels of red-cell membrane proteins and digested pallidin. Lane 1: intact pallidin; lane 2: pallidin digested at a trypsin/pallidin weight ratio of 1:250; lane 3: pallidin digested at a trypsin/pallidin weight ratio of 1:50. Digestions were carried out as described in Materials and Methods. The molecular masses of the principal digest products are shown on the right. (B) Phosphorylation of pallidin by PKA and RBC kinase and tryptic digestion. Autoradiograms of pallidin phosphorylated with [γ-^{32}P]ATP in the presence or absence of either PKA catalytic subunit or RBC kinase followed by digestion with trypsin as described under Materials and Methods. Lanes 1 and 2: autophosphorylation of RBC kinase and PKA, respectively; lane 3: autophosphorylation of pallidin pool a (no contaminating kinase activity); lanes 4 and 5: pallidin pool a phosphorylated by RBC kinase and PKA, respectively. Note the presence of the band corresponding to autophosphorylated PKA in lane 5. Lanes 6–11: digestion of pallidin phosphorylated by RBC kinase or PKA with the indicated weight ratios of trypsin was performed as described in Materials and Methods. The horizontal lines between lanes 6 and 7 show that the major phosphopeptides generated after tryptic digestion of pallidin phosphorylated by RBC kinase co-migrate with phosphopeptides generated after PKA phosphorylation. The lines between lanes 7 and 8 show that the extra phosphopeptides generated by PKA labeling correspond to PKA or its tryptic fragments. (C) Phosphopeptide domain map of pallidin. The structural domain map was derived from the N-terminal sequencing of the digested pallidin fragments shown in part A of the figure as described in Materials and Methods. The principal digest products are designated by their molecular masses, and correspond to the labeled bands in part A of the figure. This map is also used to show the locations of phosphorylated tryptic peptides, which are indicated with asterisks. Peptides were identified as being phosphorylated in two ways. In the case of the 14-, 17- and 35-kDa fragments, pallidin was phosphorylated in the presence of RBC kinase, trypsinized at an enzyme to protein ratio of 1:250 and transferred onto PVDF membrane as described under Materials and Methods. The PVDF membrane was subjected to autoradiography to identify fragments containing ^{32}P. Those fragments were subjected to N-terminal amino-acid sequence analysis which enabled the exact placement of the fragments within the tryptic domain map as shown. The 35- and 40-kDa fragments were identified as being phosphorylated by their co-migration with major ^{32}P-containing fragments under a variety of electrophoresis conditions in several experiments. The 23-kDa fragment could not be unambiguously designated as either phosphorylated or not due to its proximity to other bands.

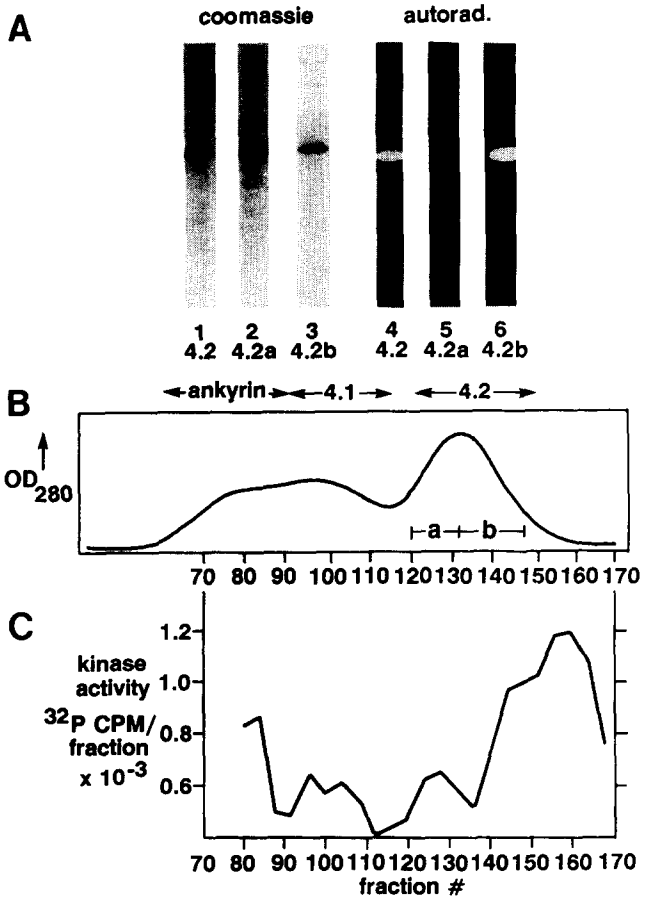

Fig. 4. (A) Phosphorylation of pallidin (band 4.2) pools from Sepharose 6B (2 M Tris) column. Pallidin pools purified from Sepharose 6B column, phosphorylated by [γ-^{32}P]ATP as described in Materials and Methods, were electrophoresed on 10% SDS-acrylamide gel followed by autoradiography. No kinase other than that which was already within the column pools was added. Lane 1: sample of entire pallidin pool from 2 M Tris column (corresponds to pools a and b in B); lane 2: leading edge of pallidin peak from 2 M Tris column (pool a in B); lane 3: trailing edge of pallidin peak from 2 M Tris column (pool b in B); lanes 4–6: corresponding autoradiographs of lanes 1–3. (B) A_{280} profile of pallidin elution from Sepharose 6B column. Labels above the fractions indicate the principal components of the indicated fractions. It should be noted that the column was eluted with 300 ml of buffer prior to fraction No. 1, and that the fraction size is 3 ml. a and b refer to the subdivision of the pallidin fractions. (C) Kinase activity of 2 M Tris column fractions. Fractions from the gel filtration chromatography of crude pallidin on Sepharose 6B in 2 M Tris were assayed for kinase activity as described under Materials and Methods. Since the fractions assayed for kinase activity contained proteins other than the added histone (e.g., pallidin) the phosphorylating activity is described simply as kinase activity rather than as histone kinase activity. In other experiments the column fractions to be assayed were electrophoresed on an SDS gel and ^{32}P counts specifically incorporated into histone were determined by cutting out and counting ^{32}P in the histone band. The results obtained from this analysis were nearly identical to that shown in the figure.

the two kinases was the same (compare lane 6 with 7 and 9 with 10). Three of the principal ^{32}P-labeled phosphopeptides which co-migrated with major Coomassie blue-staining bands (approximate molecular masses of 47 kDa, 17 kDa and 14 kDa, shown in Fig. 3B, lane 6) were subjected to N-terminal sequence analysis. Fig. 3C shows that the approx. 17- and 14-kDa peptides both start at amino acid 75, while the 47-kDa peptide starts at amino acid 246. (The peptides identified as having incorporated ^{32}P are indicated with asterisks.) Phosphorylated peptides of 35 and 40 kDa were placed in the domain map by virtue of their co-migration in several separate experiments with peptides of known origin (determined by N-terminal sequencing). Although we did determine that the C-terminal peptides of 25 and 30 kDa are not phosphorylated, it was not possible to unambiguously determine whether the 23-kDa peptide was phosphorylated.

The information in Fig. 3 localizes the phosphorylated domain(s) between amino acids 75 and 425. In order to localize the domain further, we searched the pallidin sequence [13,14] for a PKA consensus sequence RRXS [26]. We found only one such sequence in the entire protein, located at serine 248 (RRGS^{248}; numbering system of Korsgren et al. [13]). While this sequence would be included in the 47-kDa peptide, it could only be included in the approx. 14- and 17-kDa peptides if they are larger than estimated from our gel calibration. In order to include serine 248 and start at amino acid 75 the smaller of the peptides would have to be approx. 19.5 kDa and the larger approx. 21.5 kDa. While there is no guarantee that the RBC kinase is phosphorylating the same serine as PKA, results presented below show that the RBC kinase has properties consistent with it being a membrane-associated form of PKA.

The RBC kinase phosphorylated purified pallidin in solution in a time-dependent manner, and maximal phosphorylation required as much as 50–60 minutes. In two separate experiments the maximal molar incorporation of PO_4 into pallidin was between 0.3 and 0.4 mol PO_4/mol pallidin. This ratio suggests that the pallidin was already partially phosphorylated, or that not all of the pallidin is a substrate for the kinase. Control experiments showed that phosphorylated pallidin lost less than 10% of its $^{32}PO_4$ during a 3-h dialysis (at pH 7.5) following phosphorylation, suggesting that the low phosphorylation stoichiometry was not due to contaminating phosphatases which might have been active at the pH at which phosphorylation was performed.

To determine whether pallidin is a substrate for the RBC kinase when it is bound to the red-cell membrane we added RBC kinase to inside-out vesicles. Fig. 5 shows that the kinase phosphorylated all of the major proteins on the vesicles (lane 2), although pallidin is phosphorylated only weakly. Addition of a small amount of purified pallidin to the vesicles prior to phosphorylation increased their pallidin content only slightly (Fig. 5 lane 3, bottom) but resulted in a signifi-

cant increase in pallidin phosphorylation (Fig. 5 lane 3, top). A similar result was obtained following addition of pallidin to inside-out vesicles which had been stripped of all endogenous pallidin (Fig. 5 lanes 4, 5 and 6). These results show that purified pallidin reconstituted onto red-cell membranes is a good substrate for the RBC kinase. Similar results were also obtained with PKA (not shown).

The above results suggest that purified pallidin may be a better substrate for phosphorylation than endogenous membrane-associated pallidin. One possible explanation for this phenomenon is that membrane associated pallidin is already phosphorylated, while the purified protein is de-phosphorylated, possibly as a result of the purification procedure. To test this hypothesis we pre-treated inside-out vesicles containing endogenous pallidin with alkaline phosphatase prior to phosphorylation with the RBC kinase. Fig. 6 shows

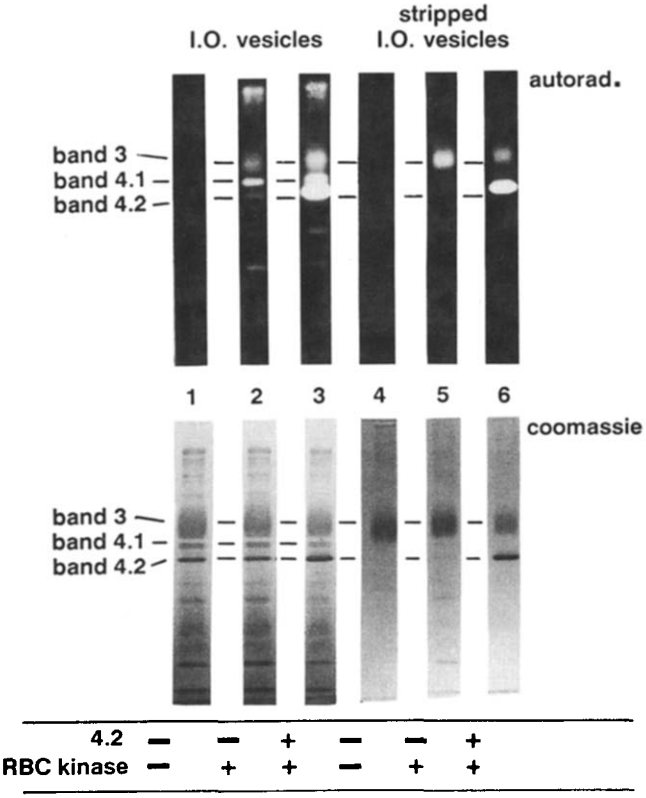

Fig. 5. Phosphorylation of pallidin (band 4.2) added to inside-out and stripped inside-out vesicles. Top: autoradiogram. Bottom: Coomassie blue. Inside-out vesicles were reconstituted with purified pallidin and phosphorylated with RBC kinase as described in Materials and Methods. Lane 1: inside-out vesicles incubated with [γ-^{32}P]ATP and no added kinase; lane 2: inside-out vesicles phosphorylated by RBC kinase; lane 3: inside-out vesicles reconstituted (see Materials and Methods) with 50 μg/ml of purified pallidin and phosphorylated by RBC kinase; lane 4: pH 11-stripped inside-out vesicles incubated with [γ-^{32}P]ATP and no added kinase; lane 5: pH 11-stripped inside-out vesicles phosphorylated by RBC kinase; lane 6: pH 11-stripped inside-out vesicles reconstituted with 50 μg/ml purified pallidin and phosphorylated by RBC kinase. The major phosphorylated bands are labeled.

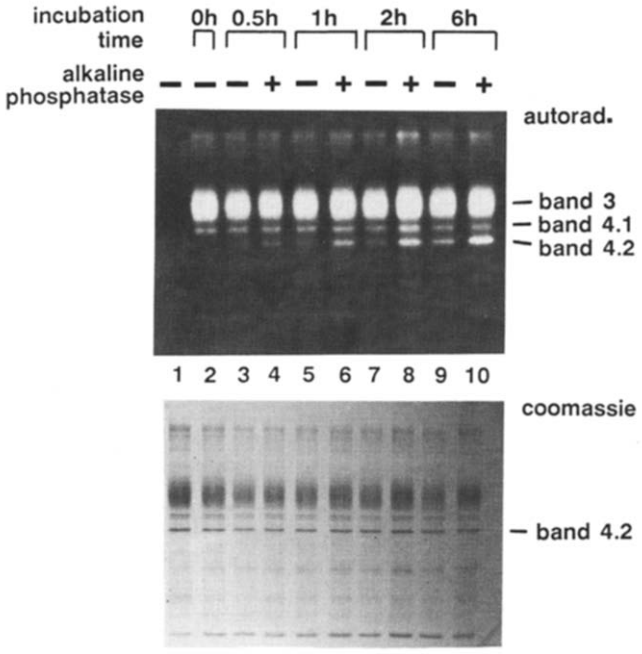

Fig. 6. Alkaline phosphatase treatment of inside-out vesicles. Top: autoradiogram. Bottom: Coomassie blue. Inside-out vesicles were incubated with or without alkaline phosphatase as described under Materials and Methods for the times indicated. Lane 1: inside-out vesicles not treated with alkaline phosphatase were incubated with [γ-^{32}P]ATP in the absence of added kinase as described in Materials and Methods; lane 2: inside-out vesicles were placed in phosphatase treatment buffer but without added phosphatase and immediately phosphorylated by the addition of RBC kinase and [γ-^{32}P]ATP; lanes 3–10: inside-out vesicles were pre-incubated with or without alkaline phosphatase, as indicated, for 0.5–6.0 h and then phosphorylated by addition of RBC kinase and [γ-^{32}P]ATP as described in Materials and Methods. The major phosphorylated bands are labeled (band 4.2: pallidin).

that the phosphatase treatment led to an increase in the phosphorylation of pallidin but in no other protein (compare lanes 7 and 8 for example). This suggests that membrane associated pallidin may contain endogenous phosphate or be inaccessible to the kinase.

The RBC kinase was found to phosphorylate a variety of Histones but was particularly active on Kemptide, a peptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) which is a relatively specific substrate for PKA kinase (not shown). The kinase activity was not stimulated by cGMP, cAMP, calcium plus calmodulin or phosphatidlyserine (not shown). The lack of significant stimulation by cAMP indicates that the RBC kinase as purified here is not regulated by cAMP, however, the preference for Kemptide as a substrate indicates that it may contain activity similar to the cAMP-independent catalytic subunit of PKA. We compared the substrate specificity of the RBC kinase with PKA catalytic subunit using Histones I, II, III and VI. When adjustments were made for enzyme-specific activity, PKA and the RBC kinase had similar substrate specificities (not shown). The one notable exception is Histone Type VI,

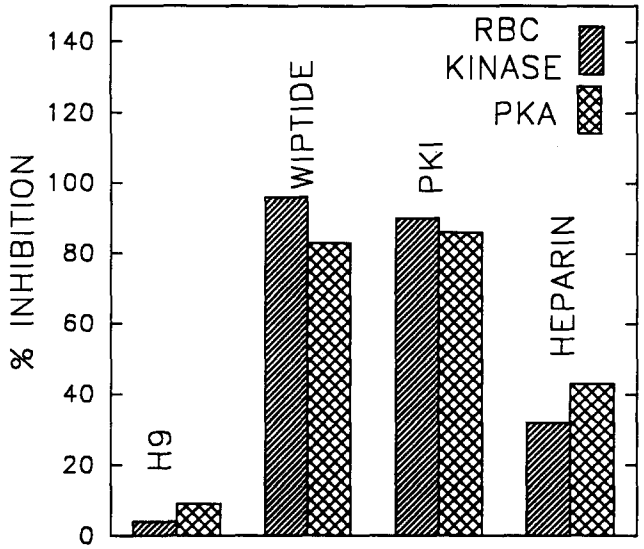

Fig. 7. Effect of kinase inhibitors on RBC kinase and PKA catalytic subunit. Kinase activity was assayed as described in Materials and Methods using 10 μg of Histone type II as substrate. The concentrations of the different inhibitors used are as follows: H9, 2.5 μM; Wiptide, 50 μM; PKI, 10 μg; Heparin, 6 μg/ml. Each of the inhibitors shown were tested in at least two separate experiments, with similar results. The data shown are the means of duplicate assays, which agreed to within $\pm$15%.

which is a much better substrate for the RBC kinase than for PKA.

To further compare the RBC kinase with PKA catalytic subunit we examined the effects of various kinase inhibitors on their activities. Fig. 7 shows that when Histone Type II is used as a substrate the isoquinoline sulphonamide protein kinase inhibitor H9 was unable to inhibit either kinase when used at a concentration at which it is reported to inhibit PKA [27]. However, phosphorylation of Histone Type III by both PKA and RBC kinase could be inhibited by H9 (75% and 58% inhibition, respectively), although Histone type III is not as good a substrate as histone type II for either kinase (data not shown). Fig. 7 also shows that heparin, a potent inhibitor of casein kinase II, has only a small inhibitory effect on the kinases. Significantly, PKI, a heat stable inhibitor with high specificity for PKA, inhibited both kinases 80–90%, as did Wiptide, (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-I le-NH_2) another highly specific peptide inhibitor of PKA.

Discussion

The method described here for the purification of pallidin yields a protein that has physical characteristics which are different from the protein purified by our previously published method. (For convenience of discussion we will refer to pallidin purified by our previous method as Type I pallidin and that purified by the current method as Type II pallidin.) The principal difference between the two types of pallidin relate to their solubility. The Type I protein remains in solution under a variety of conditions, including moderate salt concentrations (up to at least 0.12 M KCl). By contrast, although the Type II protein was stable in low salt (20 to 50 mM KCl), it formed sedimentable aggregates in the presence of 0.1 M KCL or NaCl. It should, however, be noted that even the Type I protein suffers from variable non-specific sedimentation from preparation to preparation (C.M. Cohen and C. Korsgren, unpublished data). Physical characterization of the Type II protein was not possible since the protein aggregated in sucrose gradients and stuck to the top of gel filtration columns. We found that limiting the KCl concentration to 20 mM and including 0.25% ovalbumin, and 0.05% Tween-20 reduced aggregation and non-specific sedimentation of the protein.

The limited solubility of the Type II protein makes it unsuitable for certain types of studies such as physical characterization. On the other hand, type II pallidin is considerably purer than Type I (> 97% pure vs. 85–90%) making it more useful for digestion and mapping studies such as that shown in Fig. 3, as well as for chemical analysis. For example the Type II protein was the one used to demonstrate that erythrocyte pallidin is N-myristoylated [28]. It is tempting to speculate that this N-terminal myristate plays an important role in targeting pallidin to its membrane binding site, as is the case for the *src* kinase for example (reviewed in Ref. 29). Moreover, it is also possible that the myristate has a strong effect on the solubility properties of pallidin discussed above. If the myristate does play a role in determining the solubility properties of pallidin, it will be interesting to determine whether Type I and Type II pallidin have different myristate contents.

Suzuki et al. [30] have shown that pallidin on red-cell membranes can be phosphorylated only when Cd^{2+} or Hg^{2+} were added. The role played by these cations was not clearly defined, but it was speculated that they might bind to sulphydryl groups in pallidin, possibly leading to the exposure of previously inaccessible phosphorylation sites. Similarly, Fennell et al. [31] demonstrated the phosphorylation of erythrocyte pallidin both in the presence of Zn^{2+} and at alkaline pH, conditions which they hypothesized would activate endogenous phosphatases and permit subsequent phosphorylation by [γ-^{32}P]ATP. We have identified a red-cell membrane-associated kinase which co-extracts and partially co-purifies with pallidin and avidly phosphorylates pallidin in solution. By comparison, when this kinase is added to inside-out vesicles it phosphorylates endogenous pallidin poorly. One possible explanation for this is that membrane-associated pallidin is fully phosphorylated while purified pallidin is at least partially dephosphorylated. This proposal is consistent with the

hypothesis of Fennell et al. [31] and is supported by our observation that pre-treatment of inside-out vesicles with alkaline phosphatase leads to an enhancement in the subsequent phosphorylation of pallidin by the RBC kinase.

If pallidin in inside-out vesicles is accessible to exogenous alkaline phosphatase why don't its phosphate groups undergo turnover in the red cell, which contains cytosolic phosphatases? One possibility is that the perturbation of the membrane, and elution of spectrin and actin, involved with the production of inside-out vesicles promotes the accessibility of the pallidin to the phosphatase. Alternatively, it is possible that the pH 8.5 buffer in which the phosphatase is used has some effect on the accessibility of the phosphorylated domains.

Pallidin is not normally phosphorylated during metabolic $^{32}PO_4$ labeling of erythrocytes or when [γ-^{32}P]ATP is added to ghosts (reviewed in Ref. 32). There are several possible explanations of why the purified protein can be phosphorylated in solution or when added back to inside-out vesicles. One is that the site which is phosphorylated by RBC kinase is occupied by PO_4 in the native protein on the membrane, and that this PO_4 undergoes no turnover during $^{32}PO_4$ metabolic labeling of intact cells and is not lost during preparation of ghosts. This suggests the possibility that pallidin is phosphorylated prior to or at the time of its assembly onto the membrane, and that phosphorylation may play an important role in regulating the associations of pallidin with other proteins. An alternative explanation is that the phosphate acceptor sites are more accessible in the purified protein than in the native protein, possibly because the purified protein is partially denatured or has assembled onto the membrane incorrectly.

Our experiments suggest that the kinase which partially co-purifies with pallidin is similar or identical to the catalytic subunit of PKA. Significantly, Suzuki et al. [30] also presented evidence that the kinase which phosphorylated pallidin in the presence of Cd^{2+} or Hg^{2+} was PKA. Red cells are known to contain a species of PKA which is tightly associated with the membrane, requiring detergents or elevated pH for solubilization [33–35]. Since the RBC kinase characterized here is extracted from the membrane under harsh conditions (2 M Tris) it also has the property of being tightly membrane associated. Membrane-associated kinases frequently co-purify with red-cell membrane or membrane skeletal proteins. For example, band 4.1 purified by the method of Tyler et al. [36] contains a contaminating kinase [37] and band 4.9 (dematin) co-purifies with a kinase which is similar or identical to the catalytic subunit of PKA [38]. Our data show that although the RBC kinase shares a number of properties with PKA some properties of RBC kinase are distinct from those of PKA. Most notably, RBC kinase shows no evidence of autophosphorylation, which is seen prominently in PKA. In light of the above it is most likely that the RBC kinase preparation contains multiple kinase activities and that contaminating kinases other than PKA are responsible for some of the distinctions noted above.

At this time, we have no indication that this phosphorylation affects any of the known interactions of pallidin. However, our data are consistent with the hypothesis that pallidin may be phosphorylated during, or shortly after, synthesis and membrane association, and that this phosphate undergoes little or no turnover in the mature red cell. This phosphorylation might therefore be important in stabilizing the pallidin conformation, or its association with other proteins.

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