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Phosphorylation sites in human erythrocyte band 3 protein

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The human red cell anion-exchanger, band 3 protein, is one of the main phosphorylated proteins of the erythrocyte membrane. Previous studies from this laboratory have shown that ATP-depletion of the red blood cell decreased the anion-exchange rate, suggesting that band 3 protein phosphorylation could be involved in the regulation of anion transport function (Bursaux et al. (1984) Biochim. Biophys. Acta 777, 253–260). Phosphorylation occurs mainly on the cytoplasmic domain of the protein and the major site of phosphorylation was assigned to tyrosine-8 (Dekowski et al. (1983) J. Biol. Chem. 258, 2750–2753). This site being very far from the integral, anion-exchanger domain, the aim of the present study was to determine whether phosphorylation sites exist in the integral domain. The phosphorylation reaction was carried out on isolated membranes in the presence of [γ-^{32}P]ATP and phosphorylated band 3 protein was then isolated. Both the cytoplasmic and the membrane spanning domains were purified. The predominant phosphorylation sites were found on the cytoplasmic domain. RP-HPLC analyses of the tryptic peptides of whole band 3 protein, and of the isolated cytoplasmic and membrane-spanning domains allowed for the precise localization of the phosphorylated residues. 80% of the label was found in the N-terminal tryptic peptide (T-1), (residues 1–56). In this region, all the residues susceptible to phosphorylation were labeled but in varying proportion. Under our conditions, the most active membrane kinase was a tyrosine kinase, activated preferentially by Mn^{2+} but also by Mg^{2+}. Tyrosine-8 was the main phosphate acceptor residue (50–70%) of the protein, tyrosine-21 and tyrosine-46 residues were also phosphorylated but to a much lesser extent. The main targets of membrane casein kinase, preferentially activated by Mg^{2+}, were serine-29, serine-50, and threonine(s)-39, -42, -44, -48, -49, -54 residue(s) located in the T-1 peptide. A tyrosine phosphatase activity was copurified with whole band 3 protein which dephosphorylates specifically P-Tyr-8, indicating a highly exchangeable phosphate. The membrane-spanning fragment was only faintly labeled.

Introduction

The possibility that reversible membrane protein phosphorylation controls erythrocyte membrane stability and various functional properties has been recently reviewed by Boivin [1]. This regulatory mechanism depends on the activity of protein kinases and phosphatases located in the membrane and in the cytosol. The human red cell anion exchanger, band 3 protein, is one of the main phosphorylated proteins of the erythrocyte membrane.

However, the precise functional role of the phosphorylation of the erythrocyte membrane proteins is still unclear [2]. Recent reports on the phosphorylation of ankyrin and protein 4.1, two skeletal proteins, suggest that it reduces their interaction with band 3 protein (3–7). As these proteins mediate the interaction between the skeleton and the integral part of the membrane, phosphorylation could participate in the normal rheological properties of the red blood cell. By contrast, band 3 protein phosphorylation does not seem to inhibit the interaction with ankyrin [5].

Band 3 protein is made of two distinct domains: (i) a membrane spanning segment, catalyzing the exchange of anions across the membrane, and (ii) a cytoplasmic segment which is the locus of multiple interactions between the integral domain of the protein, the membrane skeleton and various cytosolic proteins, hemoglobin and glycolytic enzymes [8]. The demonstration

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; TPCK, L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone; TFA, trifluoroacetic acid; *p*-CMB, *para*-chloromercuribenzoate; *p*-NPP, *para*-nitrophenylphosphate.

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that anion transport across the red cell membrane is modulated by ATP concentration led us to postulate that ATP may act primarily through the phosphorylation of band 3 protein [9].

Previous reports have demonstrated that band 3 protein phosphorylation occurs on serine and threonine residues resulting from the activation of cytosolic and membranous casein kinases [10]. It was recently shown, at least in vitro, that band 3 protein is also phosphorylated by a specific membrane tyrosine kinase leading to the major portion of its phosphorylation [11]. Characterization of the sites of action of the various kinases [12] and of the phosphorylation of band 3 protein suggested that it occurs mainly on the cytoplasmic domain [12,13]. Only one site of phosphorylation has been localized, ascribed to tyrosine-8, close to the N-terminal end of the cytoplasmic domain [11]. With an exogeneous tyrosine kinase extracted from beef thymus Low et al. [14] showed the possible binding of a second phosphate on a tyrosine residue of the isolated cytoplasmic fragment.

We differentiated the effects of the protein kinases on band 3 protein by utilizing the preferential activation of casein kinases by Mg^{2+} and of tyrosine kinases by Mn^{2+} and showed that the protein is phosphorylated to the same extent in both conditions, but that the relative proportion of the various residues phosphorylated differs with phosphorylation conditions [15]. The recent report of the primary sequence of band 3 protein deduced from cDNA [16, 17] allowed us to establish the tryptic peptide profile of the cytoplasmic fragment of the band 3 protein [18] and of the whole band 3 protein, and to determine the precise sites of phosphorylation of this protein. At least 80% of the in vitro phosphorylation occurs on the N-terminal end of the protein, within the 56 first residues. A small percentage of ^{32}P is recovered in the membrane spanning domain, likely at the N- and C-terminal of this segment, both being cytosolic.

Material and Methods

Preparation of the red blood cells

Venous blood from ten healthy volunteers was drawn on heparin and immediately processed. The red blood cells were washed as previously described [9]. Metabolic depletion of the red cells was achieved by incubating for 20 h the washed red cells in Tris-buffered saline (pH 7.4) at 37°C. Chloramphenicol (25 μg/ml) was added to prevent bacterial growth [9].

Phosphorylation of ghosts

Red cell membrane ghosts were prepared as previously described [15] by hypotonic lysis in sodium phosphate (5 mM), EDTA (1 mM), in the presence of antiproteinase (PMSF 0.1 mM) . Membrane caseine-kinase and tyrosine kinase remain in the ghosts, allowing to perform the phosphorylation reaction without adding purified kinases. The phosphorylation reaction was performed in a 30 mM Hepes buffer (pH 7.0), by incubating the ghosts for 10 min at 30°C with [γ-^{32}P]ATP 0.01 mM (37 GBq/mmol) (Amersham), 10 mM Mg^{2+}, in the presence of 0.03 mM orthovanadate. Some experiments were performed using 2 mM Mn^{2+} as the kinase activator. At the end of the phosphorylation reaction the sample was placed on ice and diluted with 6 vol. of ice-cold buffer without [γ-^{32}P]ATP, and containing 0.03 mM orthovanadate, 15 mM *p*-NPP and 10 mM NaF to inhibit the phosphatases.

Purification of band 3 protein and its cytoplasmic and membrane spanning fragments

Band 3 protein was purified by an automated procedure as described previously [19]. Briefly, integral proteins were solubilized from the membrane in the presence of 0.5 % Triton X-100, then applied on an anion-exchange DE52 cellulose column. Band 3 protein was eluted together with other proteins with 150 mM sodium phosphate, 150 mM NaCl (pH 7.5), and was further purified on a thiol-exchange affinity column (*p*-CMB ethylagarose 4B). The band 3 protein, eluted from the affinity gel by 15 mM β-mercaptoethanol, was 95% pure and devoid of contaminating phospholipids and glycophorins. Protein concentrations were determined by the method of Lowry.

The cytoplasmic fragments of band 3 protein were purified according to Bennett and Stenbuck [20] with minor modifications [18]. The ghosts (usually 50 ml, 4 mg protein/ml) were depleted of spectrin and actin and converted to inside-out vesicles by incubation at 37°C for 30 min in 10 vol of 0.5 mM EDTA (pH 8.0). Bands 2.1, 4.1 and 6 were removed by incubation in 0.17 M acetic acid for 20 min at 24°C. Depleted inside-out vesicles were suspended in 20 vol. of 7.5 mM sodium phosphate (pH 7.5), and digested with α-chymotrypsin (1 μg/ml suspension) for 45 min. The cleavage was performed in the presence of 10% methanol at −5°C and yielded two fragments, 43 kDa and 41 kDa, in a 90/10% proportion [8]. The reaction was stopped by adding 1 mM PMSF. The cytoplasmic fragment of band 3 protein was then adsorbed on 15 ml DE52 cellulose equilibrated in a 7.5 mM sodium phosphate (pH 7.5) buffer, added directly to the membrane suspension. The resin was packed down by low speed centrifugation and poured in a column (1.5 × 25 cm). The cytoplasmic fragment was eluted from the column by a linear gradient of KCl from 0 to 500 mM in 7.5 mM phosphate buffer (pH 7.5) (50 ml/50 ml) with a flow rate of 0.2 ml/min. Using this procedure 10–12 mg of pure cytoplasmic domain was recovered.

The membrane-spanning domain was purified as described by Oikawa et al [21]. The cytoplasmic domain

was cleaved by trypsin (5 μg/ml) directly on red blood cell ghosts (50 ml). Skeletal proteins were removed by treatment with 10 vol. of ice-cold 2 mM EDTA (pH 12.0). Solubilization of integral proteins was achieved by 1% Triton X-100 and the membrane-spanning domain was isolated by chromatography on DEAE CL-6B (6 ml in a column 1.6 × 10 cm). It was eluted with a linear gradient of sodium phosphate from 5 to 250 mM (pH 8.0) (100 ml/100 ml). The whole procedure was performed in the presence of 25 mM β-mercaptoethanol. The membrane-spanning domain was eluted as a broad peak between 60 and 100 mM sodium phosphate. Using this procedure 10 mg of membrane-spanning domain was recovered. Glycophorin A was eluted at 200 mM sodium phosphate.

When phosphopolypeptides were isolated, a cocktail of phosphoprotein phosphatase inhibitors (0.03 mM orthovanadate, 15 mM *p*-NPP and 10 mM NaF) was added to all buffers except for the step of extraction of skeletal proteins which requires a low ionic strength buffer. In that case orthovanadate only was added.

Study of band 3 protein phosphorylation

SDS-PAGE was carried out on mini vertical slab gels (90 × 70 mm, 0.75 mm thick) with a 5–15% linear acrylamide gradient using the discontinuous buffer system of Laemmli. 5–20 μg of proteins were applied on each gel slot. Gels were stained with Coomassie R250 brilliant blue, dried and exposed 4, 24 and 72 h to a Kodak X-Omat AR film, using intensifying screens. Autoradiographs of the gels were allowed to develop at −80°C.

To measure phosphate covalently bound, 5–10 nmol of isolated band 3 protein were precipitated with trichloroacetic acid 15% (w/v). The pellet was mineralized in the presence of 0.1 ml 70% perchloric acid at 200°C for 1 h, dissolved in 0.1 ml water and the phosphate measured with the malachite green method of Kallner [22].

Analysis of phosphoamino acids was performed after hydrochloric acid hydrolysis for 2 h, at 100°C, in HCl (constant boiling, Pierce). The lysate was dried and analyzed by electrophoresis/chromatography on cellulose thin-layer plates as described [24]. The phosphorylated residues were revealed by ninhydrin staining and autoradiography. The spots were scraped out of the plates, dissolved in ACS scintillating mixture (Amersham) and the radioactivity counted (miniRack beta 1212, LKB, Sweden).

Localization of band 3 protein phosphorylation sites

(1) Limited tryptic cleavage of band 3 protein and of its cytoplasmic domain. Band 3 protein, kept in the elution buffer of the thiol-affinity chromatography (0.5% Triton X-100, 7.5 mM sodium phosphate, 15 mM β-mercaptoethanol (pH 7.4)) was digested by trypsin 10 min at 0°C using an enzyme to the substrate ratio of 1% (w/w). Tryptic digestion was stopped by adding PMSF (1 mM). Lyophilized cytoplasmic fragment was dissolved in 50 mM ammonium bicarbonate buffer (pH 7.4) and digested similarly.

(2) Tryptic digestion and structural analysis of peptides. Isolated cytoplasmic fragment of band 3 protein (3–5 mg) dissolved in 50 mM ammonium bicarbonate buffer (pH 8.8), was digested overnight at room temperature with trypsin using an enzyme to the substrate ratio of 3% (w/w). The peptides were separated by RP-HPLC using a C8 column (Aquapore RP-300, Brownlee Laboratories, Santa Clara, CA) with 7 μm particle size. Peptides were eluted with a flow rate of 1 ml/min, using a gradient between solvent A (TFA 0.05% in water) and solvent B (TFA 0.1% in water/acetonitrile, 50:50).

Whole band 3 protein and its membrane domain were digested similarly with trypsin in their own elution buffer adjusted to pH 8.8, and still containing Triton X-100 and β-mercaptoethanol. Since we observed that band 3 protein was co-purified with a tyrosine-phosphatase activity, its tryptic hydrolysis was performed in the presence of the cocktail of phosphatase inhibitors described above. The peptides were separated by RP-HPLC, using an elution gradient between solvent A (TFA 0.05% in water) and a solvent B with a higher content of acetonitrile (TFA 0.2% in water/acetronitrile, 25:75). The flow rate was 1 ml/min.

The elution of the chromatographies was followed at 214 nm and the fractions were either collected manually for further structural studies or by a fraction collector for radioactivity counting.

For determining their amino-acid composition, the peptides (0.5 nmol) were dried and hydrolyzed under vacuum for 22 h at 110°C in the presence of 30 μl HCl (Pierce constant boiling). Amino-acid analyses were performed by RP-HPLC, after conversion of the amino-acids into phenylthiocarbamyl (PTC) derivates, according to Heinrikson and Meredith [23] using a C18 column (Brownlee Laboratories Spheri-5 μ 250 × 4.6 mm). The chromatograms were recorded at 254 nm, memorized and computerized using a Shimadzu C-R5A integrator. Peptides were sequenced on a gas phase Sequencer (Applied Biosystems, model 470 A).

(3) Characterization of the phosphorylation. 1 ml fractions were collected during the HPLC separation of the peptides and analyzed for radioactivity by Cerenkof counting.

For further characterization of the phosphorylation sites within the N-terminal peptide (peptide T-1) of the protein, which contains 56 residues, T-1 was submitted to cyanogen bromide cleavage. The peptide (1–2 nmol) was dissolved in 50% formic acid and cyanogen bromide was added in a ratio 1:1 (w/w). The reaction was carried out for 18 h in the dark, at room temperature

and stopped by diluting with 5 vol. of water and freeze-drying. The resulting fragments were separated by RP-HPLC as described above.

Radiolabeled phosphoamino-acids were characterized in the peptides by autoradiography after electrophoresis/chromatography.

The presence of P-Tyr in the peptides of the cytoplasmic domain of band 3 protein was also demonstrated by immuno-affinity chromatography, using an anti P-Tyr affinity gel. A Sepharose gel with bound monoclonal antibodies (phosphotyrosine antibody, mouse monoclonal, clone IG2, Amersham)[24] was equilibrated in a 1 ml chromatography column with a buffer made of 10 mM Tris (pH 7.4), 5 mM EDTA, 50 mM NaCl. The tryptic digest of the cytoplasmic domain was dried under vacuum and dissolved in the same buffer before being circulated through the column for 16 h at 4°C. The unbound material was analyzed by RP-HPLC for peptide content and the radioactivity profile was determined as described above. The bound peptides were eluted from the column with 1 mM phosphotyrosine and analyzed similarly.

Peptide nomenclature

In order to localize the peptides on the linear sequence of whole band 3 protein [16, 17], the sequence was divided according to the expected tryptic cleavages. These peptides were denominated T-1 to T-75 from the N to the C-terminal of the protein, as indicated in Table I. When unusual cleavages were observed within an expected tryptic peptide, the nomenclature of the fragment refers to this last one (e.g., T-24$_1$ and T-24$_2$ corresponding respectively to residues 305–333 and 334–340).

Results

Amount of phosphate covalently bound to band 3 protein

Phosphate covalently bound to band 3 protein was measured on purified band 3 protein (i) isolated from fresh red blood cells, (ii) prepared after metabolic depletion of the cells, and (iii) after phosphorylation of the membranes in the presence of either Mg^{2+} or Mn^{2+}. The bound phosphate, expressed in mol per mol of band 3 protein, was respectively: 0.52 ± 0.08 ($n = 4$), 0.23 ± 0.03 ($n = 3$), and 0.89 ± 0.08 ($n = 6$).

Despite an identical level of phosphorylation obtained in the presence of Mg^{2+} or of Mn^{2+}, the analyses of the phosphoamino-acids labeled with ^{32}P showed a qualitative difference. When the phosphorylation reaction was activated by Mg^{2+}, P-Tyr, P-Ser and P-Thr accounted respectively for $51 \pm 8\%$, $34 \pm 5\%$ and $15 \pm 2\%$ of all phosphoamino-acids. When the phosphorylation reaction was activated by Mn^{2+}, P-Tyr, P-Ser and P-Thr accounted respectively for $79 \pm 9\%$, $17 \pm 6\%$ and $5 \pm 3\%$ of all phosphoamino acids.

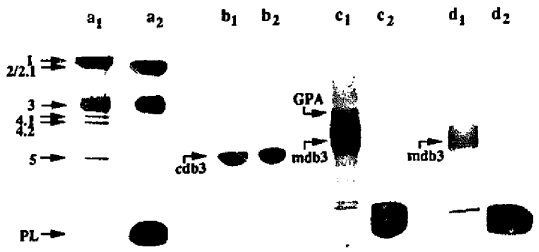

Fig. 1. Phosphorylation of red cell band 3 protein. Localization on the two domains of the protein. The phosphorylation reaction was carried out on red cell ghosts prepared by hypotonic lysis of washed red cells, by incubating 10 min at 30°C in 30 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 0.03 mM orthovanadate and 0.01 mM [γ-^{32}P]ATP (37 GBq/mmol). The reaction was stopped by diluting with ice-cold 30 mM Hepes buffer (pH 7.0), containing 0.03 mM orthovanadate, 15 mM *p*-NPP and 10 mM NaF. The cytoplasmic domain of band 3 protein was then isolated after chymotrypsic cleavage of band 3 protein on inside out vesicles according to Bennett and Stenbuck [20] with orthovanadate 0.03 mM added to all the buffers. The membrane-spanning domain was obtained after tryptic hydrolysis of the ghosts in the presence of phosphatase inhibitors, and purified according to Oikawa [21]. SDS-PAGE was carried out with a 5–15% linear acrylamide gradient using the buffer system of Laemmli. After staining with Coomassie Blue, the gels were dried and exposed to a Kodak X-Omat AR film during 4 h at −80°C, using intensifying screens. Coomassie blue stained electrophoregram of (a_1) red cell membrane proteins, (b_1) cytoplasmic domain of band 3 protein, (c_1) ghost proteins after cleavage by trypsin, (d_1) purified membrane-spanning fragment of band 3 protein. (a_2, b_2, c_2, d_2) are the corresponding autoradiograms. The following amounts of proteins were applied: lane a, 10 μg; lane b, 10 μg; lane c, 20 μg; lane d, 5 μg. The erythrocyte membrane proteins are numbered according to Fairbanks. PL, phospholipids; cdb3, cytoplasmic domain of band 3 protein; GPA, glycophorin A; mdb3, membrane domain of band 3 protein.

Localization of phosphorylated residues on the cytoplasmic domain

Electrophoresis studies and autoradiographies. In order to identify which residues are phosphorylated within band 3 protein, it was first important to determine if only one or the two domains of the protein are concerned by phosphorylation. When the red blood cell ghosts were phosphorylated by [γ-^{32}P]ATP 0.01 mM (37 GBq/mmol) in the presence of 10 mM $MgCl_2$ their analysis by SDS-polyacrylamide electrophoresis and autoradiography showed that only three components were labeled, spectrin β chain, band 3 protein and phospholipids (polyphosphoinositides) (Fig. 1a$_1$, a$_2$).

After α-chymotrypsin treatment of inside-out vesicles, both the 43 kDa and the 41 kDa fragments were labeled by ^{32}P (Fig. 1b$_1$, b$_2$).

The membrane-spanning domain of band 3 protein was recovered after limited in situ tryptic cleavage of the ghosts [21]. It was the main component of the electrophoretic pattern of skeletal proteins-depleted ghosts (55 kDa) after protein staining but was only faintly labeled (Fig. 1c$_1$, c$_2$). After chromatographic

purification of this domain on DEAE-Sepharose nearly all the radioactivity was found in the phospholipids (Fig. $1d_1$, d_2).

Limited tryptic cleavage of the cytoplasmic domain of band 3 protein (10 min at 0°C) brought further information: the radioactive label was chiefly found in the 20 kDa fragment which corresponds to the N-terminal part of the protein (Fig. $2d_1$, d_2). This was also found after cleaving the purified whole band 3 protein with trypsin in the same mild conditions: almost all the radioactivity was found in this 20 kDa peptide, a small fraction in the cytoplasmic domain and nearly none in the membrane-spanning domain (Fig. $2b_1$, b_2).

It appears therefore that phosphorylation is almost exclusively localized in the cytoplasmic domain. Further, the same specific activity was found for the cytoplasmic domain of band 3 protein and the whole band 3 protein ($5 \cdot 10^6$ cpm/mmol).

Tryptic peptide profile of the cytoplasmic domain of band 3 protein. After extended tryptic digestion of the cytoplasmic domain of band 3 protein, the peptides were separated by RP-HPLC. Modifications in the gradient and the use of a 7 μm instead of a 10 μm particle size column improved the separation of several peptides compared to that previously described [18], allowing for the characterization of the whole structure (Fig. 3 and Table I). All the peptides were identified by amino-acid analysis and, when required, by sequence analysis. No polymorphism was observed in this series comprising ten subjects.

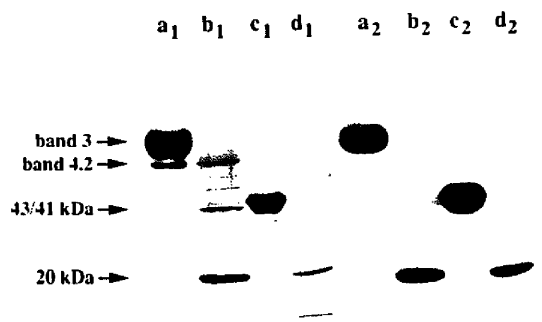

Fig. 2. Limited tryptic cleavage of band 3 protein and of its cytoplasmic domain. The phosphorylation reaction was carried out on ghosts as described in the legend of Fig. 1. Band 3 protein was extracted from the membrane by 0.5% Triton X-100 and purified by thiol exchange affinity chromatography as described in [19]. The cytoplasmic domain was purified as described in the legend of Fig 1. Band 3 protein and the cytoplasmic domain were subjected to limited tryptic hydrolysis, by reacting with trypsin for 10 min at 0 ° C using an enzyme to the substrate ratio of 1% (w/w). The reaction was stopped by adding PMSF (1 mM). SDS-PAGE was carried out with a 7.5–15% linear acrylamide gradient. Coomassie blue stained electrophoregram of (a_1) band 3 protein (b_1) fragments obtained after its limited tryptic cleavage, (c_1) cytoplasmic fragment, (d_1) fragments obtained by its limited tryptic cleavage. a_2, b_2, c_2 and d_2 are the corresponding autoradiograms. The following amounts of proteins were applied: lane a, 12 μg; lane b, 12 μg; lane c, 10 μg; and lane d, 10 μg.

^{32}P labeling. The ^{32}P-labeling was found in three radioactive peaks (Fig. 3).

Peak I, the most hydrophobic one, was eluted at 119 min together with peptide T-1. It was eluted as a double peak, denominated peaks Ia and Ib. Their amino-acid composition revealed that they were both pure T-1. Peak Ia corresponded to the principal peak of absorbancy of T-1, peak Ib was slightly more hydrophilic. Their relative proportion varied with experimental conditions.

Peak II was eluted at 99 min just before peptide T-2. However, its amino-acid composition surprisingly was identical to that of peptide T-1 (Table II). The absorbance of this peak corresponded to approx. 20% of the whole peptide T-1.

Peak III, eluted at 48 min always contained only a tiny amount of peptide and was assigned to peptide T-28–29. This peak was also eluted as a double peak. It should be noticed that peptide T-28–29 exists in two forms: the first one, resulting directly from the chymotryptic cleavage, extends from residue 347 to the Tyr-359, and is the C-terminal of the 41 kDa species, the second one is a tryptic peptide (347–360) of the 43 kDa species. Heterogeneity may also result from varying sites of phosphorylation within the peptide.

The radioactivity incorporated in peak I and peak II represented 90–94% of the total labeling. The relative proportion of peaks I and II differed with the experimental conditions for phosphorylation. When the phosphorylation reaction was carried out in the presence of Mn^{2+}, the radioactivity in peak II was higher (65%) than when it was performed in the presence of Mg^{2+} (50%), using the same membrane preparation. Peak III contained about 10% of the labeling in both conditions.

Characterization of the phosphorylated residues. After phosphorylation using Mg^{2+} as kinase activator, the radioactive peptides (peaks Ia, Ib, II, III) were hydrolyzed by hydrochloric acid and submitted to thin-layer plate electrophoresis followed by chromatography.

In peak Ia, P-Ser was the major phosphoamino acid (70%); some P-Thr (30%) and a trace of P-Tyr were also present (Fig. 4a). Peak Ib contained P-Tyr and P-Ser in comparable amount (Fig. 4b). Peak II contained only P-Tyr (Fig. 4c). In peak III, P-Tyr was the main species identified (Fig. 4d).

When the reaction was performed in the presence of Mn^{2+}, only P-Tyr was present in peak Ia (result not shown) and peaks Ib, II, and III contained the same phosphoaminoacids as in the previous condition.

Characterization of P-Tyr residues, after phosphorylating in the presence of Mn^{2+}, was also achieved by analyzing the immunospecific binding of tryptic peptide digest on an antiphosphotyrosine affinity gel. In this

last method, the tryptic digest was circulated overnight through a 1 ml column. The unbound peptides were analyzed by RP-HPLC and showed that peak II was selectively retained on the affinity column (Fig. 5A). The bound peptides were eluted with phosphotyrosine and analyzed: the main radioactive peak recovered was peak II. In addition, a part of peak I was also released, confirming that peak I is heterogeneous, containing also a phosphotyrosine (Fig. 5B). Radioactive peak III was not completely retained by the phosphotyrosine affinity column. It should be noted that peptide T-28–29 contains 2 tyrosine residues and 4 serine residues.

Localization within peptide T-1 of the sites of phosphorylation. Peptide T-1 is 56 residues long. The determina-

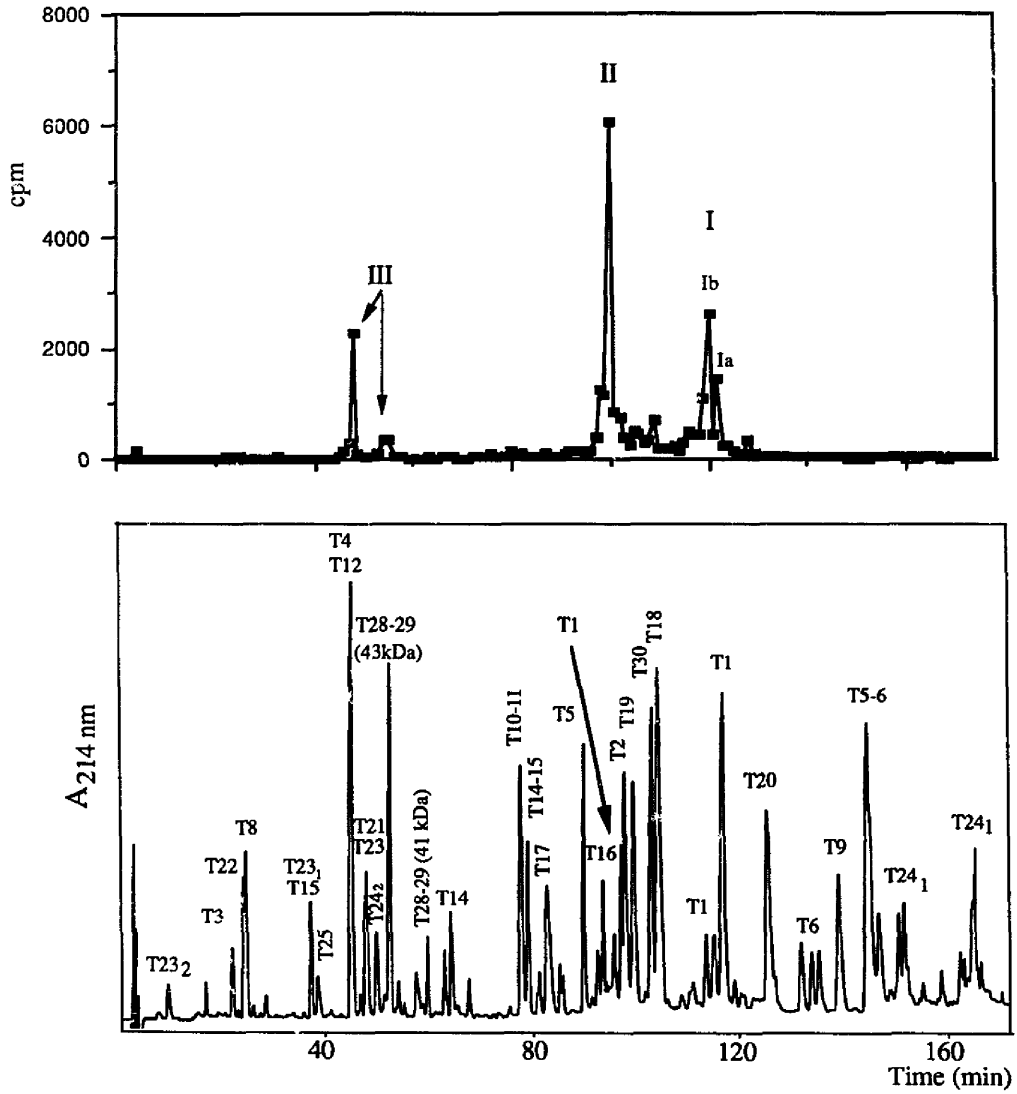

Fig. 3. Elution pattern and phosphopeptide map of the tryptic digest of the cytoplasmic domain of band 3 protein. The phosphorylation reaction was carried out on red cell ghosts, and the cytoplasmic domain of band 3 protein isolated as described in the legend of Fig. 1. 0.5 mg tryptic digest was applied onto a C8 column (Aquapore RP 300). The following gradient between solvent A (TFA 0.05% in water) and solvent B (TFA 0.1% in water/acetronitrile, 50:50) was used: 0 to 45% solvent B from 0 to 90 min, 45 to 60% solvent B from 90 to 140 min, 60 to 70% solvent B from 140 to 155 and, 70 to 100% solvent B from 155 to 170 min. The flow rate was 1 ml/min and 1 min fractions were collected and analyzed for radioactivity by Cerenkov counting. The absorbance trace at 214 nm is shown on the lower panel, and the phosphopeptide map on the upper panel. Radioactive peak I corresponds to peptide T-1. Radioactive peak II is eluted as a small peptide just before peptide T-2, as indicated by the arrow (lower panel). Amino-acid analysis revealed that it is also peptide T-1. Peak III was eluted as a double peak (arrows on the upper panel).

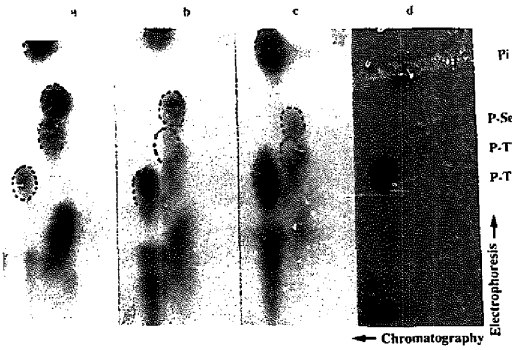

Fig. 4. Phosphoamino-acid composition of the phosphopeptides of the cytoplasmic domain of band 3 protein. The phosphopeptides separated by RP-HPLC as described in Fig. 3 were hydrolyzed for 2 h with hydrochloric acid at 100°C, in 6 M HCl ; the phosphopeptides (peaks Ia, Ib, II, and III of Fig. 3) were analyzed by double-dimension electrophoresis/chromatography on thin-layer cellulose plates. Standards were detected with ninhydrin aerosol and the ^{32}P-labeled amino acids by autoradiography. (a), peptide T-1 from peak Ia; (b), peptide T-1 from peak Ib; (c), peptide T-1 from peak II; (d) peak III.

tion of the sites of phosphorylation of T-1 required a further cleavage: cyanogen bromide reaction yielded three fragments corresponding to sequences 2–11, 13–31 and 32–56 ; each of them contains a tyrosine residue (Table II). The three species of T-1 peptide, corresponding to radioactive peaks Ia, Ib, and II, were analyzed.

The elution pattern of the cyanogen bromide-fragments resulting from peaks Ia and peak Ib was identical (Fig. 6). Hydrolysis of peak Ia showed that most of the label was contained in T-1 (32–56): Ser-50 appears therefore to be the major site of phosphorylation of this fragment. In peak Ib the labeling is mostly found in fragment T-1 (13–31) corresponding to the labeling of Tyr-21 and Ser-29.

Peak II cleavage yielded only one labeled fragment, T-1 (2–11) pointing to the phosphorylation of tyrosine at position 8. Fragment T-1(2–11) was much more hydrophilic than the corresponding fragment found after hydrolysis of peak Ia/Ib. It eluted at 38 min compared to 58 min (Fig. 7). It is very likely that the phosphorylation of the tyrosine-8 residue, adding an hydrophilic group to the highly acid cluster at the N-terminal of the molecule is responsible for the large decrease in the retention time of both the fragment T-1(2–11) and the whole peptide T-1 (peak II).

When the phosphorylation reaction was carried out in the presence of Mn^{2+}, the hydrolysis of peak Ia, which contained only P-Tyr, gave rise to two equally

TABLE I

Tryptic peptides of band 3 protein

The theoretical division in tryptic peptides was done according to the sequence of the cytoplasmic domain previously reported [18] and to the sequence of the whole protein deduced from cDNA analysis by Tanner et al. [16]. Two Lys-Pro sequences are found in the cytoplasmic domain of band 3 protein: that between peptides T14 and T15 was partly cleaved but not that between peptides T-28 and T-29. There are also two Arg-Pro sequences, at the junction T5-6 and T10-11, only the first one was cleaved but with a low yield. Besides the expected cleavages two additional ones were observed: at tyrosine-299 and at leucine-333, respectively, within peptide T-23 and T-24. n.r., peptide not recovered.

Tryptic peptide	Residues	Tryptic peptide	Residues	Tryptic peptide	Residues	Tryptic peptide	Residues
T-1	1– 56	T-21	284–292	T-38 (n.r.)	515–518	T-58	731–743
T-2	57– 69	T-22	293–295	T-39 (n.r.)	519–539	T-59	744–757
T-3	70– 74	$T-23_1$	296–299	T-40 (n.r.)	540–542	T-60 (n.r.)	758–760
T-4	75– 80	$T-23_2$	300–304	T-41 (n.r.)	543–551	T-61	761–782
T-5	81– 96	$T-24_1$	305–333	T-42 (n.r.)	552–562	T-62	783–808
T-6	97–111	$T-24_2$	334–340	T-43 (n.r.)	563–589	T-63	809–814
T-7 (n.r.)	112	T-25	341–344	T-44 (n.r.)	590	T-64	815–817
T-8	113–116	T-26 (n.r.)	345	T-45	591–592	T-65	818–826
T-9	117–138	T-27 (n.r.)	346	T-46	593–600	T-66 (n.r.)	827
T-10	139–146	T-28 [a]	347–353	T-47	601–602	T-67	828–829
T-11	147–150	T-29 [a]	354–360	T-48 (n.r.)	603	T-68	830–832
T-12	151–155	T-30 [b]	361–379	T-49 (n.r.)	604–631	T-69 (n.r.)	833–851
T-13	156–160	T-30	361–384	T-50	632–639	T-70 (n.r.)	852–870
T-14	161–174	T-31	385–387	T-51	640–646	T-71 (n.r.)	871
T-15	175–180	T-32 (n.r.)	388	T-52	647–656	$T-72_1$	872–876
T-16	181–219	T-33 (n.r.)	389	T-53	657–691	$T-72_2$	877–879
T-17	220–233	T-34	390–430	T-54	692–694	T-73	880–892
T-18	234–246	T-35	431–432	T-55 (n.r.)	695	T-74	893–901
T-19	247–263	T-36 (n.r.)	433–490	T-56	696–698	T-75	902–911
T-20	264–283	T-37 (n.r.)	491–514	T-57 (n.r.)	699–730		

[a] Peptide T-28-29 exists in two forms differing by the presence of the lysine at position 360. The first one corresponds to the C-terminal of the 41 kDa fragment and the second one to a tryptic peptide of the 43 kDa fragment. The C-terminal chymotryptic cleavage point of the 41 kDa fragment is at tyrosine-359.

[b] The C-terminal chymotryptic cleavage point of the 43 kDa fragment is within peptide T-30 at phenylalanine-379.

TABLE II

Amino-acid composition of peptide T-1 and its CNBr fragments

The theoretical composition of each peptide is given in brackets, after the measured stoichiometry. The sequence from residues 1 to 56 with the indication of the sites of cyanogen bromide cleavage is represented below the table (arrows). Residues which have been found labeled with ^{32}P are indicated in bold. The exact site of phosphorylation on threonine residues, indicated in italic, could not be ascertained.

	T1 (peak Ia) 1–56	T1 (peak Ib) 1–56	T1 (peak II) 1–56	T1 (1)	T1 (2–11)	T1 (12)	T1 (13–31)	T1 (32–56)
Asp	7.9 (8)	8.0 (8)	7.6 (8)	–	2.8 (3)	–	3.7 (3)	2.5 (2)
Glu	14.5 (16)	14.7 (16)	14.8 (16)	–	4.0 (4)	–	8.5 (9)	3.1 (3)
Ser	1.8 (2)	2.6 (2)	3.3 (2)	–	–	–	1.0 (1)	1.0 (1)
Gly	1.3 (1)	2.1 (1)	2.2 (1)	–	–	–	–	1.1 (1)
His	3.9 (4)	4.0 (4)	4.2 (4)	–	–	–	–	4.0 (4)
Arg	–	–	–	–	–	–	–	–
Thr	5.3 (6)	5.1 (6)	5.1 (6)	–	–	–	–	5.7 (6)
Ala	3.9 (4)	4.2 (4)	3.9 (4)	–	–	–	–	3.6 (4)
Pro	4.1 (4)	3.7 (4)	2.9 (4)	–	–		2.0 (2)	1.9 (2)
Tyr	2.7 (3)	1.7 (3)	1.6 (3)	–	0.8 (1)	–	0.7 (1)	0.6 (1)
Val	–	–	–	–	–	–	–	–
Met	3.0 (4)	2.6 (3)	2.3 (4)	Ac-Met	(1)	(1)	(1)	–
Ile	1.4 (1)	1.2 (1)	0.7 (1)	–	–	–	0.8 (1)	–
Leu	2.1 (2)	2.1 (2)	2.1 (2)	–	0.9 (1)	–	0.8 (1)	–
Phe	–	–	–	–	–	–	–	–
Lys	1.0 (1)	1.2 (1)	1.3 (1)	–	–	–	–	0.6 (1)
Cys	–	–	–	–	–	–	–	–
Trp	–	–	–	–	–	–	–	–

T1(1)↓ T1(2-11) 8 11↓T1(12)↓ T1(13-31) 21 29 31↓

Ac-Met-Glu-Glu-Leu-Gln-Asp-Asp-**Tyr**-Glu-Asp-Met-Met-Glu-Glu-Asn-Leu-Glu-Gln-Glu-Glu-**Tyr**-Glu-Asp-Pro-Asp-Ile-Pro-Glu-**Ser**-Gln-Met-

T1(32-56) 46 50 56

Glu-Glu-Pro-Ala-Ala-His-Asp-*Thr*-Glu-Ala-*Thr*-Ala-*Thr*-Asp-**Tyr**-His-*Thr*-*Thr*-**Ser**-His-Pro-Gly-*Thr*-His-Lys-

radioactive fragments, T-1 (12–31) and T-1 (32–56). In these conditions both Tyr-21 and Tyr-46 were phosphorylated. Peak Ib phosphorylation was not modified.

Phosphorylation of the membrane-spanning domain of band 3 protein

Structure analysis of whole band 3 and membrane-spanning domain. The elution pattern of the tryptic digest of the whole band 3 protein showed several peptides in addition to those of the cytoplasmic domain (Fig. 8, Table I). As the tryptic digestion of the whole band 3 protein and membrane domain was carried out in a buffer containing Triton X-100 and β-mercaptoethanol, these compounds are present in the chromatogram. The same additional peptides were obtained from the tryptic digest of the purified membrane-spanning domain.

The chromatographic procedure used allowed us to recover about 55% of the sequence of the membrane domain. According to the model proposed for the topography of the protein [16], most of the peptides forming loops emerging from the membrane surface were recovered, in particular peptide T-51 containing the glycosylation site at position 642, and several membrane-spanning peptides. The peptides from position 433 to 590 and from position 827 to 871, corresponding to very hydrophobic regions and both containing a Cys residue, were not recovered (Table I).

^{32}P labeling. In a preliminary experiment, we observed a complete disappearance of the label on tyrosine-8 after tryptic digestion of whole band 3 protein (Fig. 8A). This could be prevented by the addition to the sample of the anti-phosphatase cocktail during the tryptic hydrolysis (Fig. 8B) This result suggested that a specific tyrosine-phosphatase was thus co-purified with band 3 protein, as supported by results of Clari et al. [27]. By contrast, no phosphatase activity remained after purification of the cytoplasmic domain of the protein.

In the elution profile of the tryptic digest of whole band 3 protein (Fig. 8, lower panel), the ^{32}P-labeling was found in the same three peaks as those observed on chromatography of the tryptic digest of the cytoplasmic domain. An additional peak amounting to 12% of the total radioactivity (peak IV) eluted at 80 min. It should be noted that all the radioactivity of band 3 protein was dissolved, injected in the chromatograph, and recovered in these four peaks.

The tryptic digest of the membrane-spanning domain, faintly labeled on autoradiography of the electrophoresis, yielded only two radioactive peaks of similar amplitude (Fig. 9, upper panel): peak IV and a peak

eluting at the same position as the main component of peak III. The absorbance peaks, corresponding to these radioactive peaks in the elution pattern (Fig. 9, lower panel), were too minor to be analyzed. The latter peak, denominated IIIb, cannot be directly assigned to the tryptic peptide T-28–29 which was not found on the

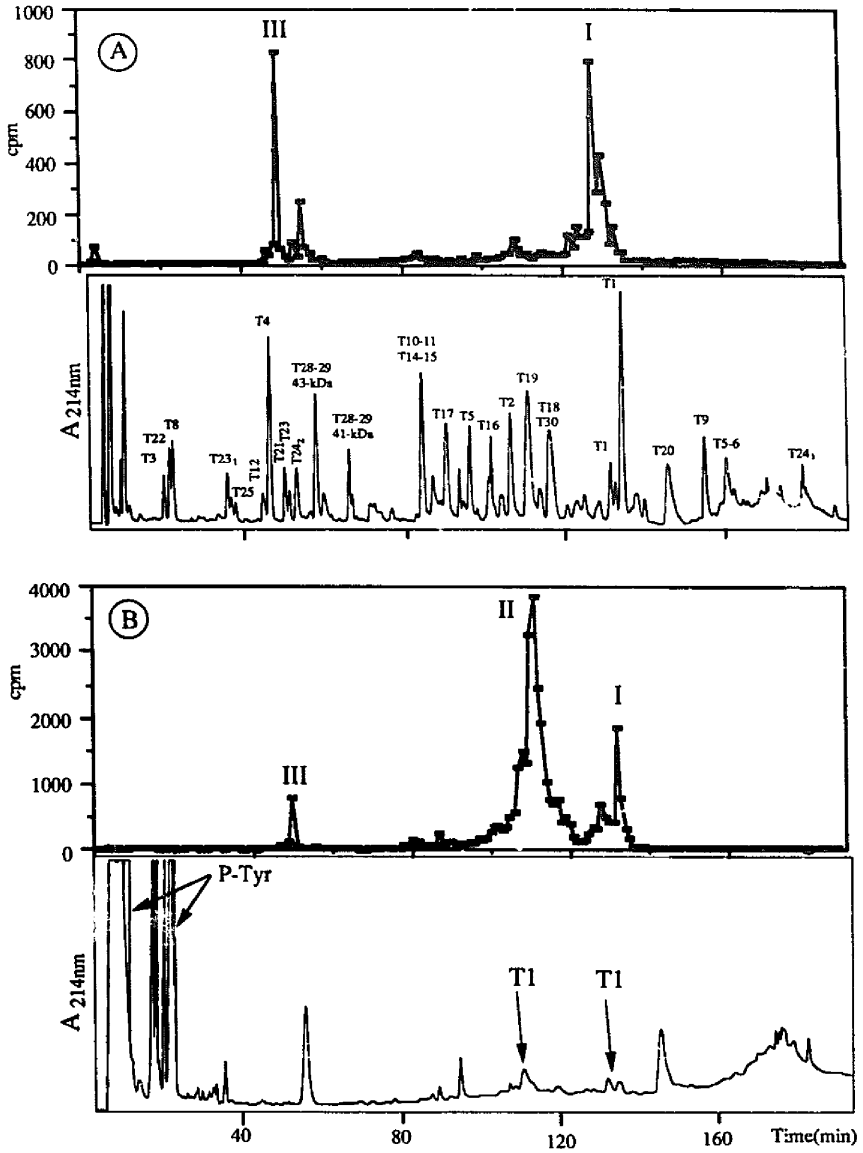

Fig. 5. Isolation of phosphotyrosyl peptides of the cytoplasmic domain of band 3 protein by monoclonal antibodies. The phosphorylation reaction was achieved on ghosts. The cytoplasmic domain of band 3 protein was then isolated and submitted to tryptic digestion. The immuno-affinity chromatography was achieved using a Sepharose gel with bound monoclonal antiphosphotyrosine antibodies and equilibrated with a buffer made of 10 mM Tris (pH 7.4), 5 mM EDTA, 50 mM NaCl in a 1 ml column. 10 mg tryptic digest of the cytoplasmic domain, dissolved in the same buffer was circulated through the column for 16 h at 4°C. The bound peptides were eluted from the column with 1 mM phosphotyrosine. Using the same experimental conditions as described in the legend of Fig. 3, the bound and unbound peptides were analyzed for peptide content by RP-HPLC, and the phosphopeptide map was determined. (A) RP-HPLC analysis of the peptides not retained by the affinity column, and the corresponding radioactive profile. (B) RP-HPLC analysis of the peptides retained by the affinity column, and the corresponding radioactive profile: almost all the labeling was recovered in the small absorbancy peaks eluted at the position of the various species of peptide T-1.

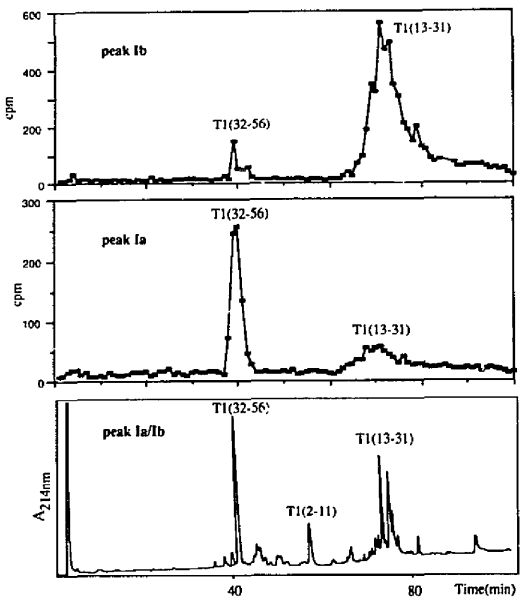

Fig. 6. Phosphopeptide map of cyanogen bromide fragments of peptide T-1 (peaks Ia, Ib). The phosphorylation reaction was achieved on ghosts, the cytoplasmic domain of band 3 protein isolated, submitted to tryptic digestion, and the phosphopeptides, separated by RP-HPLC as described in Fig. 3, were collected. Peptides T-1 (peaks Ia, Ib), dissolved in 50% formic acid, were reacted during 18 h with CNBr using a 1:1 (w/w) ratio and freeze-dried. The resulting fragments were separated by RP-HPLC, using the following gradient between solvent A (TFA 0.05% in water) and solvent B (TFA 0.1% in water/acetronitrile, 50:50): 0 to 17% solvent B from 0 to 30 min, 17 to 19% solvent B from 30 to 45 min, 19 to 22% solvent B from 45 to 55, 22 to 37% solvent B from 55 to 70 min, 37 to 50% solvent B from 70 to 80, and 50 to 100% solvent B from 80 to 100 min. The absorbance pattern at 214 nm (lower panel) was the same for both preparations. The respective radioactive profile of peaks Ia and Ib are shown.

elution pattern of the tryptic digest of the membrane domain.

Peak IV was eluted near to T-74–75, the C-terminal peptide, and may correspond to a phosphorylated form of this peptide which contains a tyrosine residue at position 904 and a threonine residue at position 894.

Discussion

The amount of phosphate covalently bound to band 3 protein depends on the metabolic status of the cell demonstrating a large decrease in phosphorylation in depleted cells. The sites of phosphorylation of the protein have been determined and the results reported here show that phosphorylation occurs mainly on the N-terminal, cytoplasmic part of the protein. A faint labeling by ^{32}P is also demonstrated on the membrane domain.

^{32}P labeling concerns primarily the N-terminal part of the cytoplasmic domain

After purification of both the cytoplasmic and the integral domains of the protein we found that most of the radioactivity was localized in the cytoplasmic region. Tryptic digestion of the cytoplasmic domain and isolation of the radioactive peptides showed that phosphorylation essentially involves residues located in the N-terminal peptide T-1.

The tyrosine at position 8 is the major site of phosphorylation, accounting for approximately half of the labeling after phosphorylating in the presence of either Mg^{2+} or Mn^{2+}. However, P-Tyr-8 is not the only target for the tyrosine kinase ; we demonstrate that both tyrosine-21 and -46 in peptide T-1 may be phosphorylated, but to a much lesser extent, whereas tyrosine-58 (in peptide T-2) was never labeled in any condition studied. The 23 N-terminal residues form two repeats of amino-acid sequences, but it has to be emphasized that tyrosine-21 is only weakly phosphorylated compared to tyrosine-8. It is likely that the end of the molecule is a better target for the tyrosine kinase. Band 3 tyrosine kinase is reversibly bound to the membrane through electrostatic interactions with the polyacidic sequence surrounding the phosphate acceptor tyrosine-8 [25]. We suggest that the high specificity of recognition requires some specific structural arrangement.

The reversible phosphorylation of band 3 protein involves also the activity of phosphatases. A highly specific tyrosine-phosphatase has been described in the human red cell cytosol. This enzyme dephosphorylates the tyrosine-phosphorylated cytosolic fragment of band 3 protein [26]. We observed that band 3 protein was co-purified with a phosphatase and that if no inhibitor was added to the preparation, radioactive peak II could not be recovered, pointing to the high specificity of the enzyme toward P-Tyr-8 (Fig. 8).

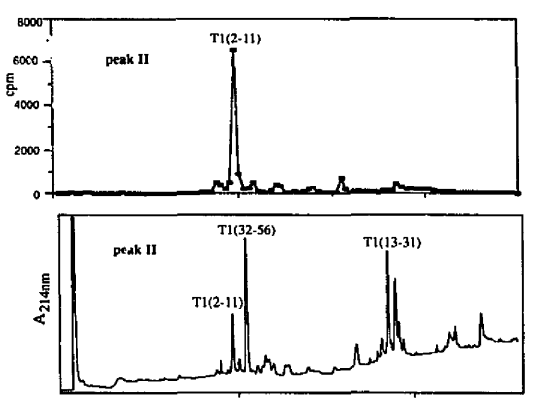

Fig. 7. Phosphopeptide map of cyanogen bromide fragments of peptide T-1 (peak II). Experimental conditions as in Fig. 6.

The rest of phosphorylation in the N-terminal peptide was distributed among serine residues 29 and 50, threonine residue(s) of the sequence 39–54. These residues are localized in a region containing a cluster of six threonine residues, realizing the target for casein kinase. Similar sites for phosphorylation have also been observed in other proteins. For example, glycophorin A dimer is phosphorylated near the C-terminal of the molecule where several hydroxyl residues are located (unpublished results and Ref. 27). Mammalian and avian β-adrenergic receptors, despite poorly conserved sequences, contain a similar serine- and threonine-rich region at or near the cytoplasmic carboxyl terminus where they are phosphorylated [28].

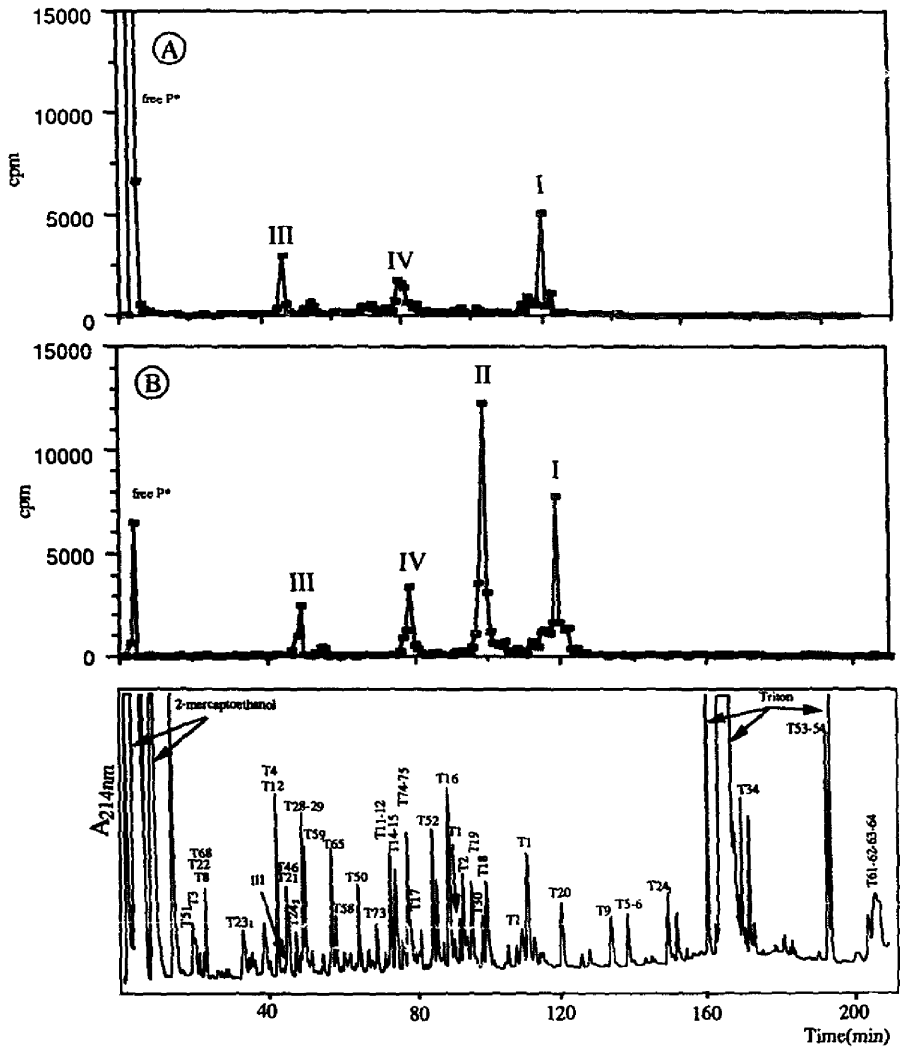

Fig. 8. Phosphopeptide map of whole band 3 protein. The phosphorylation reaction was achieved on ghosts and whole band 3 protein isolated as described in the legend of Fig 2. Whole band 3 protein was digested with trypsin in the buffer used for separation, still containing Triton X-100 and β mercaptoethanol, adjusted to pH 8.8. One mg tryptic digest was applied onto the C8 column for RP-HPLC. Elution was performed at a flow rate of 1 ml/min, using the following gradient between solvent A (TFA 0.05% in water) and solvent B (TFA 0.2% in water/acetonitrile, 25:75): 0 to 30% solvent B from 0 to 90 min, 30 to 40% solvent B from 90 to 140 min, 40 to 47% solvent B from 140 to 155, 47 to 67% solvent B from 155 to 170 min, and 67 to 100% solvent B from 170 to 220. The absorbance pattern at 214 nm is shown in the lower panel. (A) Phosphopeptide map obtained after tryptic digestion in the absence of phosphatases inhibitors. (B) Phosphopeptide map obtained after tryptic digestion in the presence of phosphatases inhibitors (0.03 mM orthovanadate, 15 mM *p*-NPP and 10 mM NaF).

Is a conformational change induced by phosphorylation of tyrosine-8 ?

It may be noticed that tyrosine-8 is in a sequence which shows some homology of charge with the loop regions of EF-hand segments of calcium binding proteins [30]. These regions display negative charges at the putative calcium coordinate x, z, $-x$ and $-z$ positions, while the $-y$ position is occupied by a tyrosine residue. The region of the molecule near tyrosine-8 has been proposed for the binding of calcium and it has been hypothesized that calcium binding to the N-terminal part of band 3 protein could induce some conformational change of the cytoplasmic domain which would decrease the interaction with ankyrin and lower the anion transport rate [30]. Phosphorylation could regulate anion transport according to a similar mechanism. A good argument for a conformational change induced by phosphorylation is provided here by the large decrease in retention time observed during RP-HPLC analysis for peptide T-1 when it is phosphorylated at tyrosine-8. This is in contrast with the very slight modification of the retention time when the phosphorylation occured at any other position. A conformational change induced by phosphorylation of the acidic cytoplasmic

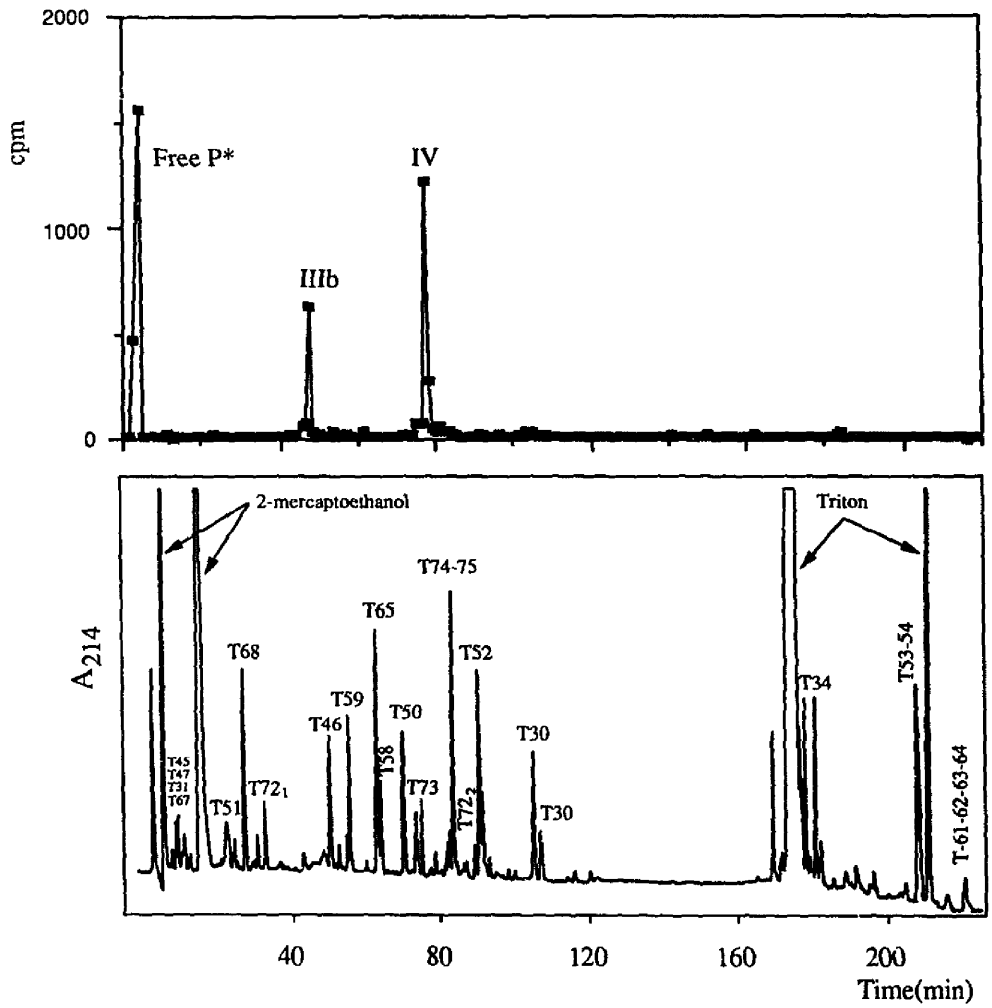

Fig. 9. Phosphopeptide map of the membrane-spanning domain of band 3 protein. The phosphorylation reaction was achieved on ghosts, and the membrane-spanning domain of band 3 protein was isolated as described in the legend of Fig. 1. Membrane domain of band 3 protein was digested with trypsin in the buffer used for its preparation, still containing Triton X-100 and β-mercaptoethanol, adjusted to pH 8.8. The peptides were separated by RP-HPLC, using the same chromatographic procedure as described in the legend of Fig. 8. The absorbance pattern at 214 nm is shown on the lower panel, the phosphopeptide map on the upper panel.

domain of band 3 protein could modify its interaction with the 'loops', rich in basic amino acids, separating the membrane-spanning α-helices [31].

The possibility for a conformational change of peptide T-1 upon phosphorylation is presently under study by NMR analysis.

Other phosphorylation sites

Among the other tyrosine residues of the cytoplasmic fragment it seems that only those of the C-terminal peptide (T-28–29) were labeled.

A small amount of radioactivity was associated with the membrane fragment of the protein, isolated after limited tryptic cleavage of the ghosts. It was distributed into two peptides. The first one is likely to correspond to the C-terminal peptide (T-75) of band 3 protein. This peptide, localized on the cytoplasmic face of the membrane, contains at the eighth position before the C-terminal a tyrosine residue in an acidic environment similar to that surrounding tyrosine-8 at the N-terminal of the molecule [16]. We were not able to identify the second radioactive peak in the membrane domain, present in a very small amount.

Physiological meaning of band 3 protein reversible phosphorylation

Contrasting with the high specificity for the in vitro phosphorylation of tyrosine-8, there was still no strong evidence for such a mechanism in vivo. Analysis of the phosphoamino acid present in band 3 protein after incubation of whole red blood cells in the presence of P_i yielded results varying with the incubation conditions. Only when incubating in an isotonic medium containing glucose and 0.03 mM orthovanadate could a significant amount of phosphotyrosine be demonstrated [29]. In our experimental conditions, the RP-HPLC analysis of the tryptic digest of the cytoplasmic domain prepared from native ghosts, with no incubation with added ATP and vanadate, revealed the presence of a small absorbance peak eluting at the same position as the tyrosine-8 phosphorylated-T-1 peptide. We showed that the displacement of T-1 on the chromatogram was specific of phosphorylation on tyrosine-8 as such a displacement was also observed on the CNBr cleaved fragment containing ^{32}P-labeled-tyrosine-8. It is therefore likely that phosphorylation on tyrosine-8 occurs in vivo. There is no such 'marker' for the other phosphorylation sites. It is likely that the phosphorylation sites described in this study after in vitro labeling may be present in vivo, but with different relative intensity. For example, adding vanadate to the reaction medium, which favors tyrosine phosphorylation, allowed to describe the sites of action of tyrosine kinase. Caseine kinase mediated phosphorylation on serines 29/50 and threonines, amounting to a third of whole phosphorylation even in these conditions, could be predominant in in vivo conditions.

The present study reveals also some phosphorylation sites close to the integral part of the protein and their faint labeling, in our in vitro conditions, does not disprove a physiological meaning. On the other hand, phosphorylation on tyrosine-8 seems to be very specifically regulated, with both the tyrosine kinase and the tyrosine phosphatase bound to the N-terminal part of the protein. A physiological role in regulating the binding (and the function hereby) of glycolytic enzymes was proposed by Low et al. [14]. A modulation of anion transport rate could also be mediated by the phosphorylation of the cytoplasmic domain. The demonstration of a maintained anion transport after cleavage of the cytoplasmic domain of band 3 protein [31] does not preclude a modulation of this transport through an interaction between the two domains of the protein. Indeed, Kopito et al. showed that the membrane domain of a neuronal homolog of erythrocyte anion-exchanger was sufficient to insure anion-exchange, but that the cytoplasmic domain could play a regulatory role in anion-exchange [32].

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