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Identification of the phosphorylation site on human erythrocyte band 7 integral membrane protein: implications for a monotopic protein structure

Ulrich Salzer [a], Horst Ahorn [b] and Rainer Prohaska [a]

[a] *Institute of Biochemistry, University of Vienna, Medical Faculty, Vienna (Austria) and* [b] *Bender & Co., Vienna (Austria)*

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Band 7 membrane protein was metabolically labelled with [^{32}P]phosphate in the presence of cAMP, isolated and digested, the labelled peptides were purified and sequenced. Ser-9 was identified as the only phosphorylation site. This proves that the N-terminal region is located at the cytoplasmic side of the membrane and implies a monotopic rather than the predicted bitopic structure.

Introduction

The major integral membrane protein of the human erythrocyte band 7 region (also termed protein 7.2b or 'stomatin') is a 31 kDa phosphoprotein. It was recently purified and partially characterized by immunochemical and proteinchemical methods [1,2]. Furthermore, cDNA clones coding for this protein were isolated from bone marrow and HeLa cell libraries; their deduced amino acid sequences (287 amino acids) were essentially identical [3,4]. Computer-analysis assigned the protein to a class of bitopic transmembrane proteins, characterized by a short exoplasmic N-terminal region, a single membrane-spanning domain and a major cytoplasmic C-terminal region [3,4].

The function of band 7 membrane protein is still unknown. However, red cells lacking this protein show a high permeability for Na^+ and K^+ [5], a fact that raises the possibility that the protein may be involved in the regulation of an ion-channel. Since this regulation is often associated with protein phosphorylation and dephosphorylation [6,7], and it is well documented that band 7 membrane protein is phosphorylated in intact cells in the presence of cAMP [1,2,8], we undertook the identification of the respective phosphorylation site. We localized it to the N-terminal region and thereby proved the cytoplasmic orientation of this domain.

Correspondence to: R. Prohaska, Institute of Biochemistry, University of Vienna, Medical Faculty, Dr. Bohr-G. 9, Room 3506, A-1030 Vienna, Austria.

Abbreviations: cAMP-PK, cAMP-dependent protein kinase; HPLC, high-performance liquid chromatography; TPCK, 1-chloro-3-tosylamido-4-phenylbutan-2-one.

Materials and Methods

Materials. Human blood was obtained from the Austrian Red Cross; sequencing grade endoproteinases and cAMP were from Boehringer, Mannheim, Germany; carrier free [^{32}P]phosphoric acid was from ICN, Irvine, CA; Immobilon-P membranes from Millipore, Bedford, MA.

Phosphorylation and isolation of band 7 membrane protein. Erythrocyte membrane proteins were metabolically labelled with [^{32}P]phosphate in the presence of 0.1 mM cAMP as described [8], except for a 50-fold increase in scale. The membranes were prepared by lysis in 5 mM EDTA, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium phosphate (pH 8.0), at 4°C, followed by centrifugation for 30 min at 48 000 × *g*. The pellet was washed twice in lysis buffer and solubilized with an equal volume of 2% SDS in lysis buffer for 3 min at 95°C. Preparative scale phosphorylation was carried out using 200 ml packed red cells and 10 mM sodium phosphate (pH 7.4). Membranes were prepared and solubilized as above; the resulting solution was combined with the ^{32}P-labelled membrane proteins, and diluted 1:20 with cold 0.35% Triton X-100, 5 mM EDTA, 5 mM NaF, 1 mM sodium azide, 5 mM sodium phosphate (pH 8.0). Solid NaCl was then added with stirring to 140 mM. Band 7 membrane

protein was affinity-purified from this solution by GARP-50-Sepharose, as described [1].

Preparation and sequencing of band 7 phosphopeptides. The purified, ^{32}P-labelled protein was subjected to preparative SDS-PAGE [9] and blotting [10] to Immobilon-P. It was visualised by Ponceau S-staining and autoradiography, excised from the blot and digested as described [11] by TPCK-trypsin or Lys-C proteinase in the buffer recommended by the manufacturer. The supernatant was acidified with formic acid and applied to a reverse-phase HPLC column (Nucleosil 300, 5 μm, C_{18}, 4 × 150 mm). Peptides were eluted at 1 ml/min using a 0 to 100% acetonitrile gradient in 0.1% trifluoroacetic acid; absorbance was measured at 214 nm. Fractions were collected and radioactivity was measured by Cerenkov counting. Sequence analysis of the radioactive peak fractions was carried out by automatic sequencing (Applied Biosystems model 477A sequenator).

Phosphoamino acid analysis. Immobilon-P-bound ^{32}P-labelled protein was subjected to gas-phase hydrolysis and the phosphoamino acids were analysed as described [12].

Results

Phosphorylation and isolation of band 7 membrane protein

Red cells were metabolically phosphorylated in the presence of 0.1 mM cAMP, using phosphate and [^{32}P]phosphate. Band 7 membrane protein was then purified from the solubilized membranes by immunoaffinity-chromatography and identified by SDS-PAGE, Coomassie blue-staining and autoradiography (Fig. 1).

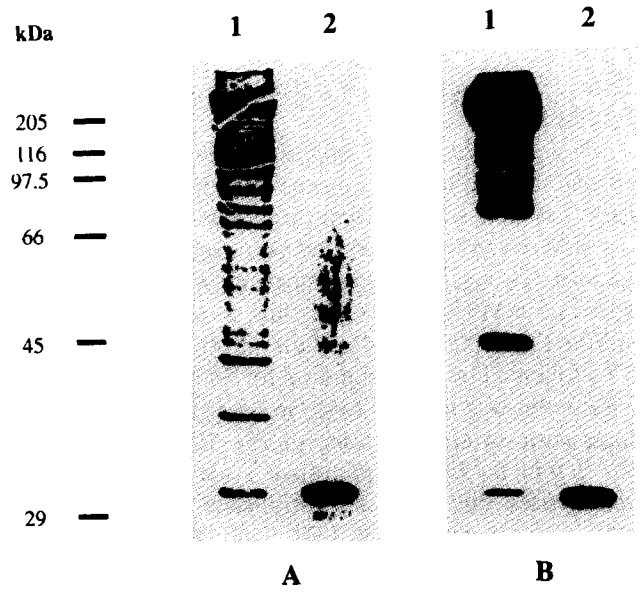

Fig. 1. Isolation of ^{32}P-labelled band 7 membrane protein. Metabolically phosphorylated erythrocyte membrane proteins (lane 1) and affinity-purified band 7 membrane protein (lane 2) were subjected to SDS-PAGE (12% acrylamide), Coomassie blue-staining (A), and autoradiography (B).

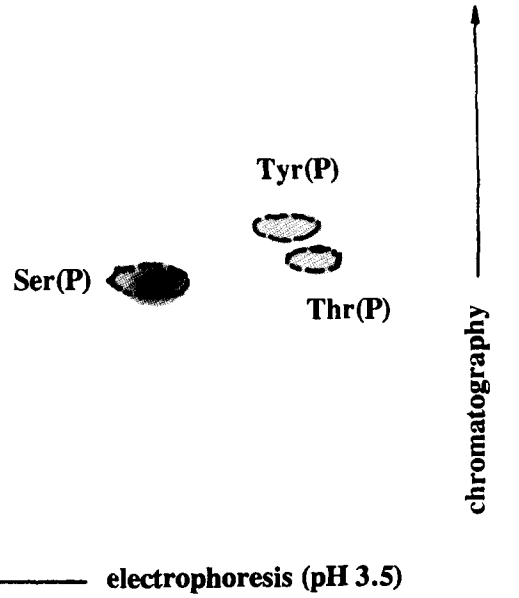

Fig. 2. Phosphoamino acid analysis. ^{32}P-labelled band 7 membrane protein was hydrolysed and the phosphoamino acids were determined by two-dimensional thin-layer electrophoresis/chromatography. Ninhydrin-positive marker spots are encircled.

Phosphoamino acid analysis

Two-dimensional analysis of hydrolysed ^{32}P-labelled band 7 membrane protein showed that phosphoserine is the only phosphoamino acid present (Fig. 2). The single ^{32}P-labelled peptide isolated from a Lys-C digest (Fig. 3) gave the same result (not shown).

Preparation and sequence analysis of phosphopeptides

Phosphorylated band 7 membrane protein was digested with trypsin or Lys-C proteinase. The peptides were separated by reversed phase HPLC (Fig. 3), and the ^{32}P-labelled peptides were sequenced. When the tryptic digest was analysed, three radioactive peaks were found. The major peak T-3 was rechromatographed under isocratic conditions, in order to purify the radioactive peptide. Its sequence corresponded with amino acids 8–24 of the N-terminal domain (Table I). Peaks T-1 and T-2 were not analysed, however, thin-layer chromatography of T-1 showed that it co-migrates with inorganic phosphate (data not shown), and T-2 may be a cleavage product of T-3.

HPLC analysis of the Lys-C digest showed only one radioactive peak (L-1), which contained 80% of the injected radioactivity; the sequence corresponded with amino acids 4–19 (Table I). Peptide L-1 contains three potential phosphorylation sites: Thr-6, Ser-9, and Ser-17; the putative cAMP-dependent site Thr-6 was ruled

out, because of the lack of phosphothreonine in band 7 membrane protein and peptide L-1 (Fig. 2). Calculating the yield of serine residues across the Edman degradation cycles showed that Ser-9 was 30% reduced, when compared to Ser-17. The decrease in Ser-9 recovery was not observed when a peptide was sequenced, that was derived from unphosphorylated band 7 membrane protein. These results indicate that Ser-9 is the phosphorylated residue. For further analysis we cleaved the peptide L-1 with Asp-N proteinase:

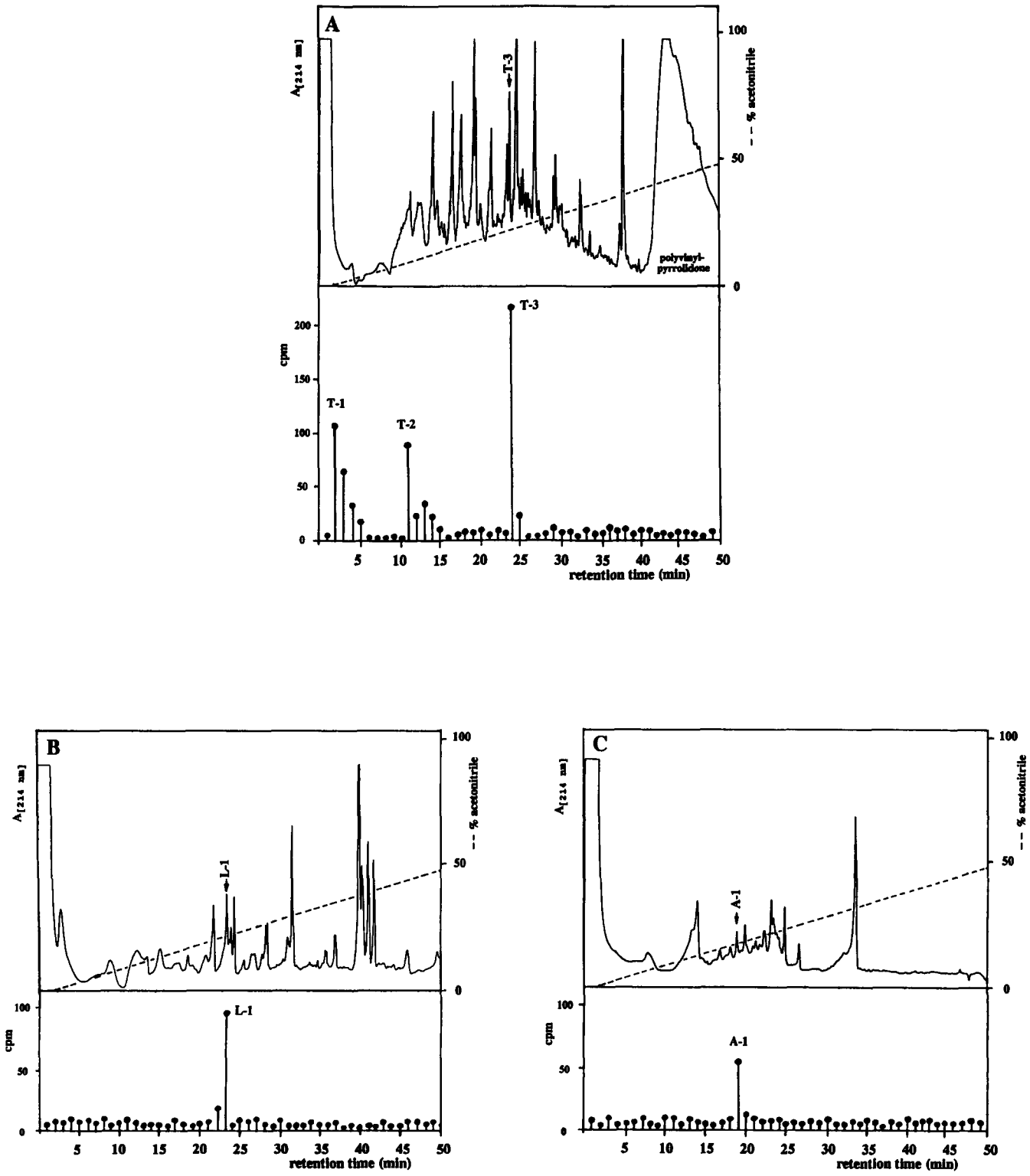

Fig. 3. Profile of HPLC-separated phosphorylated band 7 peptides. Metabolically phosphorylated band 7 membrane protein was digested with trypsin (A) or Lys-C (B) and the resulting peptides were separated by reverse-phase HPLC. Peptide L-1 was further digested with Asp-N (C).

TABLE I

Sequences of HPLC-separated ^{32}P-labelled peptides

Digest	Peak	Amino acid sequence	Residues
Trypsin	T-3	DSEAQRLPDSFKXSPSX	8–24
Lys-C	L-1	RHTRDSEAQRLPDSFK	4–19
Asp-N	A-1	DSEAQRLP	8–15

the HPLC-fractions gave only one radioactive peak (A-1), and the sequence of the peptide corresponded with amino acids 8–15 (Table I). This sequence revealed that Ser-9 is the only phosphorylated residue.

Discussion

We isolated human erythrocyte band 7 membrane protein, that has been phosphorylated in intact cells in the presence of cAMP, and identified the respective phosphorylation site as Ser-9. This site does not fulfil the typical features of a cAMP-PK site, because the sequence T-R-D-S lacks a basic residue at position −3 [13]. Nevertheless, it does appear susceptible to phosphorylation by cAMP-PK, because the sequence R-X-S/T has been described as a sufficient, though not optimal, consensus for cAMP-PK [14]. Interestingly, Thr-6, which is part of a typical cAMP-PK consensus, was not phosphorylated.

Ser-9 is located in the 24-residue N-terminal domain of the protein, predicted to face the exoplasmic side of the membrane [3,4]. However, the existence of an exoplasmic domain is questionable, because proteolysis studies have shown, that band 7 membrane protein is not degraded by proteinases (pronase and proteinase K) at the cell surface of intact erythrocytes [1,4,15]. The protein is readily digested when unsealed ghosts are used, indicating its predominant cytoplasmic localization. The C-terminus itself has been confined to the cytoplasmic side by in situ carboxypeptidase Y-digestion, followed by Western blot analysis (Mayer, H. and Prohaska, R., unpublished data), using our monoclonal antibody GARP-50 [1], which recognizes the N-terminal domain.

Digestion of unsealed ghosts, but not of intact erythrocytes, with various proteinases eventually leads to the loss of the GARP-50 epitope [1], showing that the N-terminus is in fact accessible to the proteinases at the cytoplasmic side of the membrane, but not at the exoplasmic face. The phosphorylation of Ser-9 is yet another proof of the cytoplasmic localization of the N-terminal domain, because phosphorylation of membrane proteins usually occurs at the cytoplasmic side [16] and is used as a natural marker for cytoplasmic orientation. Therefore, we conclude that band 7 membrane protein is not a bitopic transmembrane protein, but rather has a monotopic structure, being exposed at the cytoplasmic side of the membrane. There is inconsistency in the literature as to whether monotopic proteins are inserted into the lipid bilayer by a hydrophobic hairpin loop [16], or anchored only through bound lipid [17]. We can rule out by proteinchemical studies the N-terminal myristoylation and C-terminal modifications (unpublished data); however, palmitoylation of band 7 membrane protein has been described [2] and might provide the anchoring function. Since in situ hydroxylamine-treatment failed to solubilize the protein (unpublished data), we favour the hairpin-insertion model. Expression studies with mutated cysteine residues and an altered hydrophobic region, respectively, should clarify this problem. Furthermore, mutation of Ser-9 may shed light on the functional significance of the phosphorylation site.

Acknowledgements

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