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The localization of the cAMP-dependent protein kinase phosphorylation site in the platelet rat protein, rap 1B

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Rap1B is a low molecular weight G protein which is phosphorylated by cAMP-dependent protein kinase. In order to identify the site of phosphorylation by cAMP-dependent protein kinase, purified rap1B from human platelets was phosphorylated and subjected to limited proteolysis with trypsin. Single digestion fragment containing the phosphorylation site was obtained and purified by reversed-phase HPLC. Sequence analysis of the phosphorylated digestion fragment demonstrated that the sequence of the phosphorylation site was -Lys-Lys-Ser-Ser-. This sequence is near the carboxyl terminus and is adjacent to the site of membrane attachment of the protein.

Platelet; cAMP; Phosphoprotein; GTP-binding protein; Rap1B

1. INTRODUCTION

cAMP dependent protein kinase substrates and GTP binding proteins play important roles in cellular regulation. In platelets, a 22 kDa protein has been shown to be a focal point for both signal transduction systems: it is phosphorylated in either intact cells or membrane preparations by cAMP-dependent protein kinase [1–4] and it is immunologically, structurally, and functionally [5–8] identical with rap1B [9], a low molecular weight GTP binding protein that is a member of the *ras* superfamily of GTP binding proteins (also called smg-p21 [5], m22KG(I) [6], and thrombolamban [7]). Although the exact role of rap1B as a regulatory protein is not understood, phosphorylation of this and other proteins through the cAMP pathway correlates with inhibition of platelet activation [1,2,10–12].

In previous studies, we presented evidence that the amino acid in rap1B which was phosphorylated by cAMP-dependent protein kinase was a serine residue [4]. The purpose of the present studies was to identify the location of the serine phosphoamino acid in rap1B.

2. MATERIALS AND METHODS

2.1. *Materials*

Reagents, buffers, salts, and cellulose thin layer chromatography plates were obtained from Sigma Chemical Company. The catalytic subunit of cAMP-dependent protein kinase was from Sigma and had a specific activity of 30-65 picomolar u/μg where one unit was defined as the transfer of one pmol of phosphate from ATP to casein per min at pH 6.5 at 37°C. [γ-^{32}P]ATP (32 Ci/mmol) was purchased from New England Nuclear Corporation. M90, a murine monoclonal antibody raised against Ha-*ras* p21 and directed against the highly conserved GTP binding domain of the low molecular weight GTP binding protein [13], was kindly provided by Dr Juan Carlos Lacal (Madrid, Spain). Trypsin-Sepharose was prepared by linking 5 mg of TPCK-trypsin to one ml of cyanogen bromide activated Sepharose CL4B as recommended by the manufacturer.

2.2. *Rap1B purification*

Washed human platelets, prepared by differential centrifugation from outdated concentrates as described elsewhere [14], were suspended in 10 ml of 'incubation medium' (94 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.75) and sonicated for 6 10-second intervals in an ice bath using a Branson Model 200 Sonifier (Danbury, CT). Unless otherwise indicated, all subsequent steps were performed at 0–3°C. The sonicated material was then centrifuged for 10 min at 14 000 × *g* to obtain a crude membrane fraction that contained rap1B. This crude membrane fraction was suspended in 9 ml of 'incubation medium' and sodium cholate was added to a final concentration of 10% (w/v). After a 15 min incubation on ice with stirring, the mixture was centrifuged for one hour at 100 000 × *g*. The resulting supernatant, containing solubilized membrane proteins, was applied to a Sephacryl S-300 column, 2.25 × 90 cm, which was eluted with 'incubation medium' that contained 20 mM sodium cholate. Rap1B-containing fractions were pooled and protease inhibitors, phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin A, benzamidine, and EGTA, were added to final concentrations of 10 μM, 100 μM, 100 μM, 1 mM, and 1 mM, respectively. This preparation was concentrated to 5 ml of Amicon Y10 filtration and applied to a Sephacryl S-200 column, measuring 1.5 × 90 cm. The column was eluted with 'incubation medium' containing 20 mM sodium cholate and 1 mM EGTA. The rap1B fractions were pooled and rechromatographed on Sephacryl S-200 as described above. Rap1B fractions were again pooled and concentrated to 5 ml. In some preparations, further purification was achieved by hydrophobic affinity chromatography. Pooled fractions containing rap1B from the second Sephacryl S-200 step were applied to a phenyl-Sepharose column, measuring 0.9 × 5 cm, equilibrated with 'incubation medium'.

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The column was washed with 20 ml of 'incubation medium', then eluted with 40 ml of a linear 0 to 10 mM (0 to 0.67% (w/v)) gradient of Triton X-100. The phosphoprotein eluted as a symmetrical peak at approximately 6 mM Triton X-100. The resulting protein was 89–97% homogeneous by silver stained $NaDodSO_4$-PAGE gels and was 93–99% pure relative to other phosphoproteins by autoradiography.

$NaDodSO_4$-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [15] under reducing conditions on 12.5% polyacrylamide gels. Molecular mass standards were soybean trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa) and myosin (200 kDa). For Western blot analysis with the monoclonal antibody M90, proteins were electrophoretically transferred to nitrocellulose and then immunodetected with a variation of the procedure of Towbin [16] that used an alkaline phosphatase conjugated second antibody system. Autoradiography was performed by exposing nitrocellulose to Kodak XAR-5 film after immunodetection. Silver staining was performed by the method of Wray et al. [17]. Protein concentration was determined by the method of Peterson [18].

2.3. *Rap1B phosphorylation*

Purified rap1B was phosphorylated for one hour at 23°C by the addition of the catalytic subunit of cAMP-dependent protein kinase, ATP, and [γ-^{32}P]ATP for final concentrations of 2.5 units/ml, 500 mM, and 0.05 mM, respectively. After cooling on ice, unincorporated label was separated from the phosphorylated protein by chromatography on Sephacryl S-200 equilibrated with 'incubation medium' containing 20 mM sodium cholate and 1 mM EGTA as described above. In general, phosphorylation was performed after the second Sephacryl S-200 chromatography step with rap1B that was 60–70% pure. The incorporation of phosphate was approximately 0.77 mol phosphate per mol rap1B.

2.4. *Tryptic digestion, thin layer chromatography, and HPLC purification*

Phosphorylated rap1B, at a concentration of 0.25 mg/ml, was incubated with 1/10th volume trypsin-Sepharose resin for 5 min. The resin was then removed by low speed centrifugation, and the digestion mixture resolved with reversed phase HPLC chromatography utilizing a 0.46 × 25 cm Beckman ODS-Ultrasphere C_{18} column. The column was eluted at a flow rate of 2 ml/min with collection of 2 min fractions as follows. One ml of the digestion mixture containing 5–55 μg of total protein (0.23–2.5 nmol of digested rap1B) was applied per run. The column was eluted with H_2O/trifluoroacetic acid (TFA)/acetonitrile (CH_3CN) = 99.95/0.05/0 (v/v/v) for 20 min, a linear gradient of H_2O/TFA/CH_3CN = 99.95/0.05/0 to H_2O/TFA/CH_3CN = 0/0.05/99.95 for 40 min, and H_2O/TFA/CH_3CN = 0/0.05/99.95 for 5 min. The column was then returned to 0.05% TFA in H_2O with a 20 min re-equilibration period. The digestion mixture and HPLC-purified phosphopeptide were subjected to cellulose thin layer chromatography with a butanol/pyrimidine/acetic acid/water = 37.5/25/7.5/30 (v/v/v/v) solvent system. Autoradiography was performed after development of the TLC plate, then radiopeptides were scraped and quantified by liquid scintillation counting.

2.5. *Sequence analysis*

Sequence analysis of rap1B and the HPLC-purified peptides was performed on an Applied Biosystems Model 477A sequencer, using standard protocols.

3. RESULTS AND DISCUSSION

Limited tryptic digestion of phosphorylated rap1B resulted in cleavage of the 22 kDa protein into 2 fragments: a 20 kDa fragment containing the M90 epitope (Fig. 1, lane 4), and a smaller fragment that migrated with the tracking dye and was not discriminated on 12.5% acrylamide gels. Autoradiograms revealed that the phosphorylation site sequenced was contained in the smaller fragment since the 20 kDa fragment containing the M90 epitope was not radiolabeled (Fig. 1, lane 2). More prolonged exposure to trypsin resulted in further digestion of the 20 kDa fragment into 16 and 13 kDa fragments (results not shown).

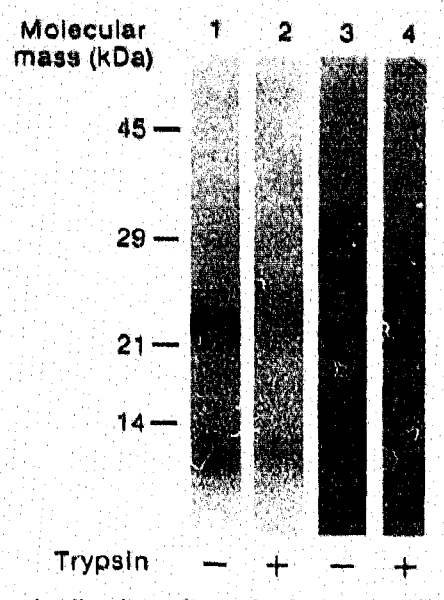


Fig. 1. Tryptic digestion of rap1B. Rap1B, purified to 89% homogeneity, was digested by the addition of solid phase trypsin. After 5 min at 21°C, the sample was centrifuged at 10 000 × g to remove the trypsin and the supernatant was mixed with an equal volume of sample buffer and electrophoresed on 12.5% acrylamide gels. Lane 1: autoradiogram of undigested rap1B. Lane 2: autoradiogram of digested rap1B. Lane 3: M90 indirect immunostain of undigested rap1B. Lane 4: M90 indirect immunostain of digested rap1B.

To isolate the small radiolabeled fragment containing the phosphorylation site sequence, labeled proteins in the digestion mixture were separated by reversed-phase HPLC (Fig. 2). The major peak of radioactivity, con-

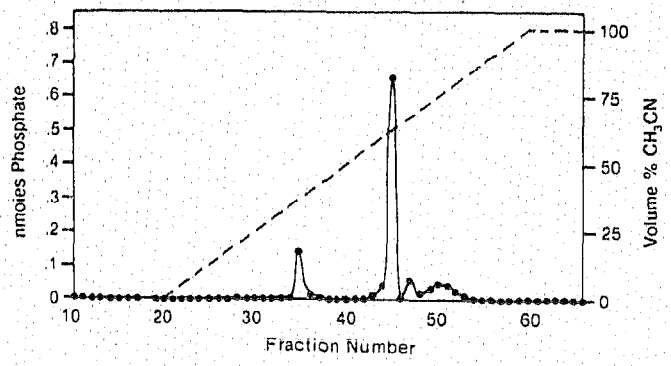


Fig. 2. Reversed phase HPLC purification of the rap1B phosphorylation site. The rap1B digestion mixture was applied to a C_{18} reversed-phase HPLC column which was subsequently developed with an acetonitrile gradient to obtain the phosphorylated fragment.

taining approximately 35% of the total radioactivity applied to the column, eluted in fraction 45. A small peak of radioactivity in fraction 34, constituting 5% of the applied radioactivity, was found by NaDodSO$_4$-PAGE and autoradiography to contain the 12 kDa impurity, depicted in lanes 1 and 2 of Fig. 1. Smaller amounts of radioactivity in fractions 46–53 contained less than 5% of the radioactivity applied to the column. The digestion mixture and fraction 45 were subjected to cellulose thin layer chromatography. The digestion mixture contained one major phosphopeptide wth an R_f value of 0.518 (71% of the applied radioactivity) and 2 minor radiolabeled fragments with R_f values of 0.311 and 0.118 (15% and 14% of the applied radioactivity, respectively). Fraction 45 contained a single phosphopeptide with a R_f value that was the same as that of the major peptide in the digestion mixture. Based on this TLC result and the radiopurity of the undigested rap1B preparation (93% based on densitometric scans of lane 4 in Fig. 1), the phosphorylated compound in fraction 45 could have originated only from intact rap1B, and not from an impurity in the predigestion sample. The 20 kDa fragment containing the M90 epitope was recovered as a broad peak between fractions 43 and 55.

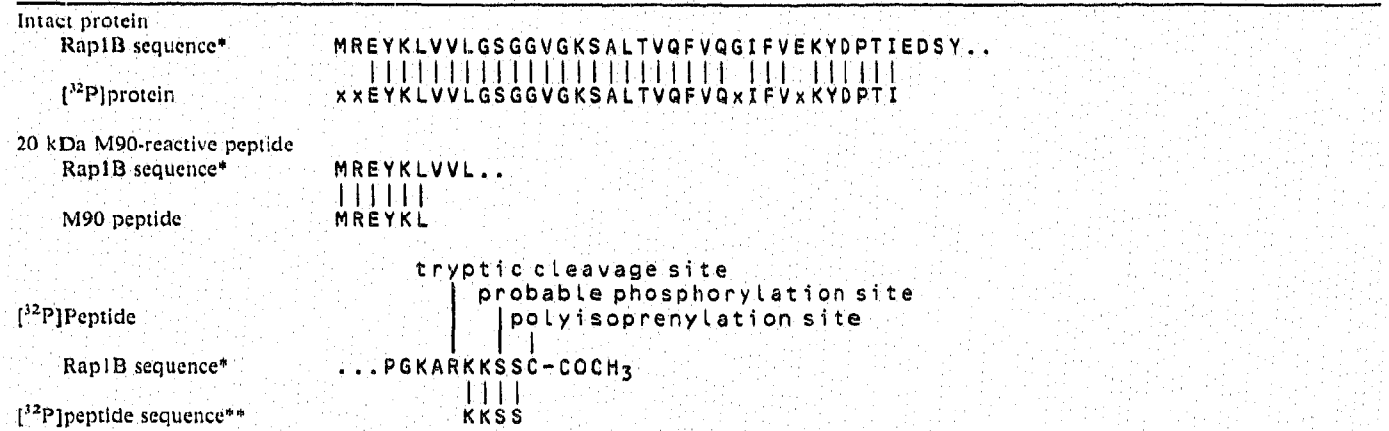
Sequence analysis of the tryptic peptides obtained from C_{18} HPLC purification as well as the sequence of the intact protein obtained from phenyl-Sepharose chromatography is presented in Table I and compared with the known amino acid sequence of rap1B deduced from the cDNA sequence [8,9]. The sequence obtained for the phosphorylated peptide was -Lys-Lys-Ser-Ser- and was identical to a unique sequence near the carboxyl terminus of rap1B and was not present in rap1, rap2, rac, ral, or any other known low molecular weight G protein sequence. The yield of serine in cycles 3 and 4 were progressively reduced, with yields of 25.4% and 9% with respect to the amount applied to the sequence and then no further sequence was obtained. A possible explanation for this may be related to the polyisoprenyl group at the C-terminal cysteine residue of rap1B [23]. Due to the hydrophobic character imparted to the peptide by the polyisoprenyl group, there would be increased washout of the peptide from the filter as the polar amino acid residues are removed during the sequencing reaction. As a result, the yield would decrease with each sequencing cycle.

We were unable to determine which serine residue in the -Lys-Lys-Ser-Ser- sequence is phosphorylated because, with each cycle of the Edman degradation, the phosphoserine ester bond is hydrolyzed to yield the serine residue and orthophosphate, which remains filter bound [24,25]. The consensus sequence for phosphorylation by cAMP-dependent protein kinase is -Arg-Arg-X-Ser- [19]. Although -Lys-Lys-X-Ser-, a sequence present in rap1 and K-*ras*, is not phosphorylated by cAMP-dependent protein kinase, -Arg-Lys-X-Ser- is an acceptable substrate but the rate of phosphorylation is ten-fold less than the rate of phosphorylation of -Arg-Arg-X-Ser- [19]. Based on this, it is possible to speculate that the first Ser in the sequence -Lys-Lys-Ser-Ser- is phosphorylate and the recognition sequence in rap1B is -Arg-Lys-Lys-Ser-. There was no other sequence in rap1B that could form a phosphorylation site for cAMP-dependent protein kinase [19].

Increasingly, the carboxyl-terminus of rap1B and

Table I

Sequence analysis of Rap1B



Purified rap1B isolated from human platelets was phosphorylated using the catalytic subunit of cAMP-dependent protein kinase. Following digestion with trypsin, the resulting peptides were resolved by reversed phase HPLC on a C_{18} column and the sequence of the isolated peptides determined. Homology between peptice and cDNA derived sequences are indicated by vertical lines. The positions of tryptic cleavage, probable phosphorylation, and polyisoprenylation sites [23] are indicated.

*Deduced from cDNA analysis [9]

**The yields for the phosphorylated peptide in picomol were: Lys, 276; Lys, 341; Ser, 127; Ser, 45.

other low molecular weight G proteins appears to be an important part of the molecule. Sequences immediately preceding the carboxyl-terminus of the different *ras*-like proteins are the variable regions of the molecules [20], and thus are likely to be responsible for the unique properties of each member of the *ras* superfamily. The carboxyl-terminal cysteine is also the site of post-translational polyisoprenylation [21–23], a modification that has been shown to be essential for membrane attachment [22]. Finally, we present evidence here that $serine_{179}$ or $serine_{180}$, near the carboxyl-terminal end of rap1B, is the site of cAMP-dependent phosphorylation. Thus, the carboxyl-terminus of *ras*-like proteins is at the same time important for common properties of the proteins such as membrane attachment and, perhaps, for unique features such as cAMP-dependent phosphorylation. In view of the proximity between the site of cAMP-dependent phosphorylation and the site of membrane attachment, it will be interesting to determine the effect of phosphorylation of rap1B on its interaction with the membrane and other functions.

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