Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6527–6531, September 1990
Biochemistry

RAP2B: A RAS-related GTP-binding protein from platelets

(RAP proteins/small molecular weight guanine nucleotide binding proteins/cyclic AMP-dependent protein kinase)

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Communicated by George H. Hitchings, June 8, 1990

ABSTRACT **A platelet cDNA expression library was screened with the monoclonal antibody M90, which recognizes a specific epitope on *RAS*-encoded p21 proteins (amino acids 107–130). DNA sequence analysis of one clone revealed that it encoded a partial amino acid sequence of a protein closely related to RAP2, which we have named RAP2B. A repeated screening of the platelet cDNA library with an internal *Ava* I fragment of the RAP2B cDNA allowed the isolation of a full-length cDNA for the *RAP2B* sequence. RAP2B is 90% identical to RAP2 at the amino acid level with the most variability at the carboxyl terminus of the protein. Oligonucleotides were synthesized to complete the amino acid sequence of the RAP2B protein and the entire sequence was expressed in *Escherichia coli*. Analysis of crude soluble extracts indicated that RAP2B was a M_r 22,000 protein that specifically bound GTP on blots. Moreover, incubation of similar extracts with the catalytic subunit of cAMP-dependent protein kinase did not cause phosphorylation of RAP2B, as had been observed for the closely homologous proteins, RAP1A and RAP1B. These results suggest that RAP2B, like the other RAP proteins, is a low molecular weight GTP-binding protein in human platelets.**

Platelets contain several small molecular weight GTP-binding proteins (M_r 21,000–31,000), some of which are related to the *RAS* oncogene product on the basis of sequence and binding of monoclonal antibodies specific to the *RAS* oncogene product p21 (1–10). One of these proteins is either RAP1A (11) or RAP1B (12); both of which have been shown to be phosphorylated *in vivo* by agents that increase cAMP levels and *in vitro* by the catalytic subunit of cAMP-dependent protein kinase (PKA). RAP1B (13) and the closely related protein RAP1A (14) [Krev-1 (15), smg-21 (16)] have been cloned from several tissues including platelets and human erythroleukemia cells and expressed in *Escherichia coli* (ref. 12 and unpublished observations).

We were interested in isolating cDNAs for other RAS-related small molecular weight GTP-binding proteins from human platelets to determine what roles they may have in platelet function related to aggregation, secretion, or both. To that end, we screened a platelet cDNA library with monoclonal antibody M90, which recognizes a highly conserved epitope of HRAS p21 involved in GTP binding (amino acids 107–130) (17). We report here on the cloning, sequence analysis,‡ expression, and preliminary characterization of a small molecular weight GTP-binding protein closely related to RAP2.

EXPERIMENTAL PROCEDURES

Library Screening. The platelet expression cDNA library in λgt11 (18) was screened with monoclonal antibody M90 essentially as described (19). Antibody incubations were as described for Western blots (8, 9). Positive plaques were purified through three rounds of screening. The same library was rescreened with a 750-base-pair *Ava* I fragment from the original clone under standard conditions (19) with positive plaques purified through three rounds of screening.

Isolation of Phage DNA, Fragment Subcloning, and DNA Sequencing. Phage DNA was purified from plate lysates by us standard procedures (19). The purified DNA was digested with *Eco*RI, the insert fragments were isolated by low-melting-temperature agarose gel electrophoresis, and the fragments were subcloned into *Eco*RI-digested pGEM-3Zf(−). The single-strand form of the recombinant plasmid was isolated by growing cells in the presence of the helper phage R408 and was purified under conditions described by the supplier (Promega). DNA sequence analysis was performed by the chain-termination procedure of Sanger *et al.* (20) using the enzyme Sequenase as specified by the supplier (United States Biochemical).

Expression of *RAP2B* in *E. coli*. The scheme of the expression of *RAP2B* in *E. coli* is illustrated below (see Fig. 2). A 750-base-pair fragment of *RAP2B* was released from pGEM-rap2b by digestion with *Ava* I and was purified by agarose gel electrophoresis. Two complementary oligonucleotides were synthesized (5′-GAATTCATGCGTGAATACAAGGTG-GTGGTGCTGGGC and 5′-CCGAGCCCAGCACCAC-CACCTTGTATTCACGCATGAATTC), which, when annealed, would provide the first 10 amino acids including the initiator methionine with an adjacent *Eco*RI site. The oligonucleotides were phosphorylated with T4 polynucleotide kinase, annealed, and ligated overnight at 14°C to the isolated *RAP2B* fragment. The reaction mixture was digested with an excess of *Eco*RI, the fragment was purified by agarose gel electrophoresis and was subcloned into *Eco*RI-digested pBS(−), positive colonies were identified, and the plasmid DNA was isolated. The *Eco*RI fragment containing the oligonucleotides was purified and ligated into *Eco*RI-digested pTX007, a T7 polymerase expression vector derived from pTX1927 (21) with the β-lactamase gene removed (W. S. Dallas, personal communication). Tetracycline-resistant colonies were screened for those with an insert, and the orientation was determined by digestion with *Bgl* I. Two clones were isolated, one with the insert in the correct orientation for expression [pTX-rap2b(+)] and one with the insert in the opposite orientation [pTX-rap2b(−)]. Purified plasmid was used to transform *E. coli* BL21(DE3) (22) for expression of RAP2B. A 50-ml culture was inoculated with a 1:25 dilution of an overnight culture containing either pTX-rap2b(+) or pTX-rap2b(−) and was grown to an OD_{600} of 0.7. The culture was induced with 0.4 mM isopropyl β-D-thiogalactoside and samples were removed at 2-hr and 4-hr intervals. Soluble extracts were prepared from cell pellets by suspension in a buffer containing 25 mM Tris·HCl (pH 7.5), 1 mM EDTA, 10% (wt/vol) sucrose, and 2 mM dithiothreitol and were then quick

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Abbreviation: PKA, cyclic AMP-dependent protein kinase; Ali, aliphatic amino acid.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X52987).

frozen in dry ice/ethanol. The samples were thawed on ice for 20 min and then in a 20°C water bath. Lysozyme was added to a final concentration of 0.1 mg/ml, and the suspensions were incubated on ice for 45 min followed by a brief incubation at 37°C and sonication. The insoluble debris was removed by centrifugation in a microcentrifuge for 15 min at 4°C.

Western Blot and GTP-Binding Analyses. Equivalent amounts of extracts from cultures containing either pTX-rap2b(+) or pTX-rap2b(−) were resolved in a 12% polyacrylamide gel containing SDS (23) and the proteins were electroblotted onto nitrocellulose (24). For Western blots, the filters were incubated in 50 mM Tris·HCl, pH 7.5/0.9% NaCl/polyethylene glycol (M_r 20,000; 1 mg/ml)/5% (vol/vol) calf serum for 1 hr at room temperature and then with a 1:200 dilution of antibody M90 in the same buffer with 20% calf serum for 1 hr at room temperature. The filters were washed for three 5-min periods in the above buffer minus the calf serum and were then incubated with an alkaline phosphatase-conjugated second antibody for 1 hr at room temperature. The blots were washed as above and immunoreactive bands were visualized with the appropriate substrates. For GTP binding, the filters were preincubated in a buffer containing 50 mM sodium phosphate (pH 7.5), 0.5 mM $MgCl_2$, 100 mM NaCl, and bovine serum albumin (1 mg/ml) overnight at 4°C and then for 15 min at room temperature in a buffer containing 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, and 0.3% Tween-20. [α-^{32}P]GTP (20 μCi; 1 Ci = 37 GBq) was added and the filters were incubated for 90 min at room temperature. For blocking experiments, unlabeled nucleotides were added at a final concentration of 0.1 mM. After the incubation, the filters were washed three times in the same buffer, air-dried, and exposed to film.

Phosphorylation of RAP2B by PKA. Equivalent amounts of extracts from cultures containing either pTX-rap2b(+) or pTX-rap2b(−) were incubated for 5 min at room temperature in an assay buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, bovine serum albumin (10 mg/ml), 1 mM dithiothreitol, and 25 μM ATP (4 μCi [γ-^{32}P]ATP) with or without 0.5 μg of the catalytic subunit of PKA. The reactions were terminated by adding SDS/polyacrylamide gel electrophoresis sample buffer and then immediately boiling. Samples of the assays were electrophoresed in 12% polyacrylamide gels containing SDS, stained with Coomassie blue, and exposed to film.

Materials. Restriction endonucleases and DNA-modifying enzymes were obtained from Bethesda Research Laboratories and Promega and were used according to the manufacturers' recommendations. The cloning vector pGEM-3Zf(−) and the helper phage R408 were obtained from Promega. Sequenase and the Bluescribe vector pBS(−) were obtained from United States Biochemical. The catalytic subunit of PKA was purchased from Sigma. All other reagents were of the highest quality available.

RESULTS

Isolation of RAP2B cDNA. To isolate small molecular weight GTP-binding proteins related to RAS from platelets, a cDNA expression library in λgt11 derived from platelet mRNA (18) was screened with monoclonal antibody M90. From 50,000 recombinant plaques, three clones that specifically bound antibody M90 were isolated and purified. One clone was a partial cDNA clone encoding RAP1B (13), one clone was a partial cDNA for *HRAS*, and the third contained a partial cDNA sequence that was highly homologous to the published sequence for *RAP2* (14). The library was rescreened with an internal *Ava* I fragment that contained most of the coding sequence homologous to *RAP2* and another cDNA clone was isolated that contained a putative complete open reading frame for the same clone. An ATG codon was found 18 nucleotides from the 5′ side of the original *RAP2B* clone and the sequence around that ATG (GCCATGA) was consistent with the Kozak consensus sequence for translation initiation of eukaryotic proteins (25). Moreover, the deduced amino acid sequence in this region was 100% identical to that of *RAP2*. The amino acid sequence of the cDNA from platelets and the comparison to RAP2 is shown in Fig. 1. The platelet protein was found to be 90% identical to RAP2 and 93% homologous if conservative amino acid substitutions were considered. Because of the similarity of the platelet protein to the published RAP2 sequence, we chose to designate this protein RAP2B. The sequence of RAP2B and RAP2 were most variable at the carboxyl terminus of the protein, which is consistent with the variability observed for the RAS-like proteins. However, the carboxyl-terminal Cys-Ali-Ali-Xaa motif (where Ali is aliphatic amino acid), the postulated signal for processing of RAS proteins for membrane anchoring (26–29), is conserved in RAP2B, suggesting that the protein may be polyisoprenylated (28). Moreover, two cysteines are positioned 3 and 4 amino acid residues upstream of the Cys-Ali-Ali-Xaa motif, suggesting possible palmitoylation (28).

Expression of *RAP2B* in *E. coli*. The sequence of the RAP2B protein suggested that it was a GTP-binding protein due to high homology in the regions associated with the binding of GTP to RAS p21 proteins. Therefore, we wanted to determine if RAP2B would bind GTP and also if it would act as a substrate for PKA since cAMP-dependent phosphorylation is an important event in platelet function. To obtain extracts with a significant amount of RAP2B, the cDNA was expressed in *E. coli* under the control of the T7 polymerase promoter (22), as illustrated in Fig. 2. To test for expression of *RAP2B*, cultures were grown into late logarithmic phase and were induced with isopropyl β-D-thiogalactoside. The cells were collected by centrifugation and were lysed by sonication and heat shock subsequent to lysozyme digestion. Soluble extracts were analyzed by SDS/polyacrylamide gel electrophoresis followed either by direct staining or Western

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RAP2B   - MREYKVVVLGSGGVGKSALTVQFVTGSFIEKYDPTIEDFYRKEIEVDSSP -50
          ************************* **********************
RAP2    - MREYKVVVLGSGGVGKSALTVQFVTGTFIEKYDPTIEDFYRKEIEVDSSP -50

RAP2B   - SVLEILDTAGTEQFASMRDLYIKNGQGFILVYSLVNQQSSQDIKPMRDQI -100
          ****************************************** **********
RAP2    - SVLEILDTAGTEQFASMRDLYIKNGQGFILVYSLVNQQSFQDIKPMRDQI -100

RAP2B   - IRVKRYERVPMILVGNKVDLEGEREVSYGEGKALAEEWSCPFMETSAKNK -150
          ******* ** ********** *****  ** ****** ********* *
RAP2    - IRVKRYEKVPVILVGNKVDLESEREVSSSEGRALAEEWGCPFMETSAKSK -150

RAP2B   - ASVDELFAEIVRQMNYAAQSNGDEGCCSACVIL -183
            ****************   *  ***** *
RAP2    - TMVDELFAEIVRQMNYAAQPDKDDPCCSACNIQ -183
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FIG. 1. Comparison of RAP2B and RAP2 amino acid sequences. The deduced amino acid sequences of RAP2B and RAP2 are compared as indicated. Stars, identical amino acids; spaces, mismatches. The numbers at the right are the amino acid number. The single-letter code amino acid code is used.

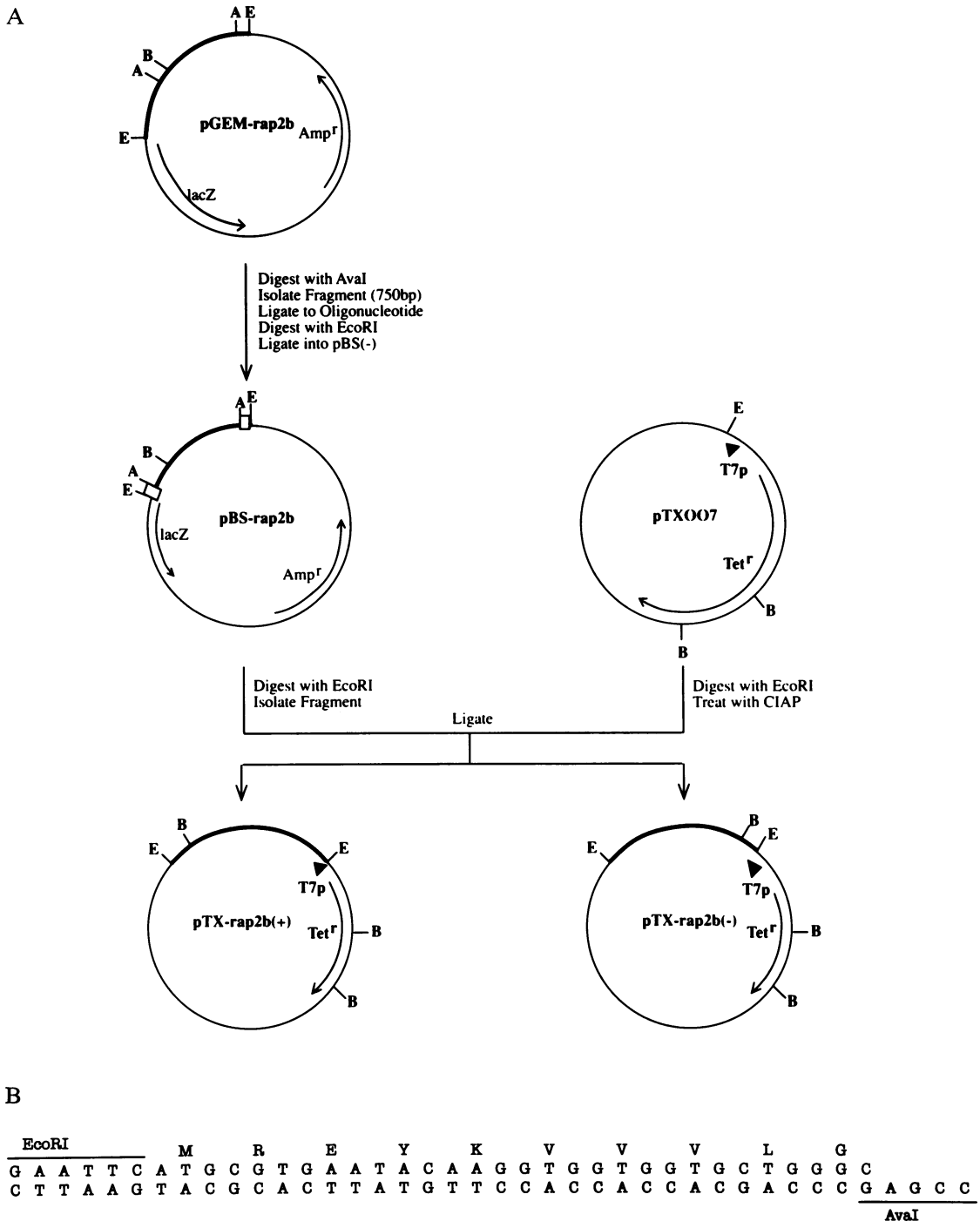

FIG. 2. Expression of *RAP2B* in *E. coli*. (*A*) Scheme for the expression of *RAP2B* in *E. coli*. Heavy line, *RAP2B* insert; open box, oligonucleotide containing the first 10 amino acids. A, *Ava* I; E, *Eco*RI; B, *Bgl* I; CIAP, calf intestinal alkaline phosphatase; T7p, T7 polymerase promoter; lacZ, β-galactosidase gene; Amp^r, ampicillin-resistance gene; Tet^r, tetracycline-resistance gene. (*B*) Nucleotide and amino acid sequence of the oligonucleotide that provides the first 10 amino acids of RAP2B with the *Eco*RI cloning site.

blot analysis (Fig. 3). Soluble extracts from cells with pTX-rap2b(+) contained a major protein band of M_r 22,000 visualized by Coomassie staining (Fig. 3, lane 1). The control cells with pTX-rap2b(−) did not contain Coomassie-staining M_r 22,000 material (Fig. 3, lane 2). Western blot analysis of the same extracts with antibody M90 showed that the M_r 22,000 protein specifically bound M90 in pTX-rap2b(+) extracts, but no M90 binding was observed in extracts from pTX-rap2b(−)-transformed cells (Fig. 3, lanes 3 and 4, respectively). Therefore, the cDNA isolated from the platelet expression library was expressed as a M_r 22,000 protein at high levels in *E. coli* under control of the T7 promoter.

GTP Binding Analysis of RAP2B. To determine if RAP2B was a GTP-binding protein, nitrocellulose blots containing soluble extracts from pTX-rap2b(+)- and pTX-rap2b(−)-transformed cells were probed with [α-^{32}P]GTP alone or with [α-^{32}P]GTP in the presence of 0.1 mM GTP, 0.1 mM GDP, or 0.1 mM ATP. Extracts from pTX-rap2b(−) cells showed no GTP binding under any conditions tested (Fig. 4, lanes 2, 4, 6, and 8). However, extracts from cells with pTX-rap2b(+) contained a M_r 22,000 protein that bound GTP (Fig. 4, lane 1). This binding was prevented if unlabeled GTP (0.1 mM) or GDP (0.1 mM) was included in the binding reaction mixture but not if ATP (0.1 mM) was included (Fig. 4, lanes 3, 5, and

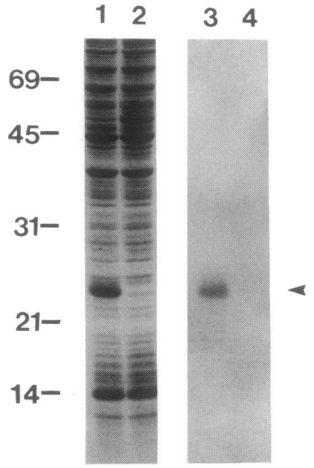

FIG. 3. Analysis of RAP2B extracts. Equal amounts (20 μl) from pTX-rap2b(+) extracts (lanes 1 and 3) and from pTX-rap2b(−) extracts (lanes 2 and 4) were applied to a 12% polyacrylamide gel containing SDS. Lanes 1 and 2 show the Coomassie blue staining profile, and lanes 3 and 4 are a Western blot analysis using monoclonal M90 as the antibody probe. Arrowhead denotes the position of the RAP2B protein. The numbers on the left are molecular weight standards ($\times 10^{-3}$): 69,000 (bovine serum albumin); 45,000 (ovalbumin); 31,000 (carbonic anhydrase); 21,000 (soybean trypsin inhibitor); and 14,000 (lysozyme).

7, respectively). No other proteins from the *E. coli* extracts bound GTP under these conditions (data not shown). These data demonstrate that the expressed RAP2B is a GTP-binding protein, as predicted by the sequence homology to *RAS*.

Phosphorylation of RAP2B with PKA. To determine if RAP2B was also a substrate for PKA, as are RAP1B and RAP1A, the extracts from both pTX-rap2b(+)- and pTXrap-2b(−)-transformed *E. coli* were incubated with the catalytic subunit of PKA, and the reaction products were resolved by SDS/polyacrylamide gel electrophoresis. Although both extracts showed phosphate incorporation into multiple proteins, none of these aligned with the expressed RAP2B protein in pTX-rap2b(+) extracts (Fig. 5). All of the phosphate incorporation was dependent on PKA, since incubation of the extracts without exogenous PKA allowed no phosphorylation under these conditions (data not shown). We conclude, therefore, that RAP2B is not phosphorylated by PKA although its possible function as a substrate for other kinases is yet to be investigated.

DISCUSSION

Screening an expression library from human platelet mRNA with monoclonal antibody M90 (17) allowed the isolation of a cDNA that encoded a small molecular weight GTP-binding protein that was 90% identical to the RAP2 cDNA cloned by Pizon *et al.* (14). Due to the high degree of homology with

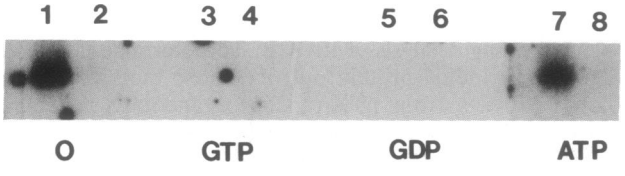

FIG. 4. GTP-binding analysis of RAP2B. Equal amounts (10 μl) from pTX-rap2b(+) (lanes 1, 3, 5, and 7) and pTX-rap2b(−) (lanes 2, 4, 6, and 8) extracts were applied to a 12% polyacrylamide gel containing SDS and were electroblotted onto nitrocellulose. The filters were analyzed for GTP-binding. 0, No addition of unlabeled nucleotide; GTP, addition of 0.1 mM GTP; GDP, addition of 0.1 mM GDP; ATP, addition of 0.1 mM ATP.

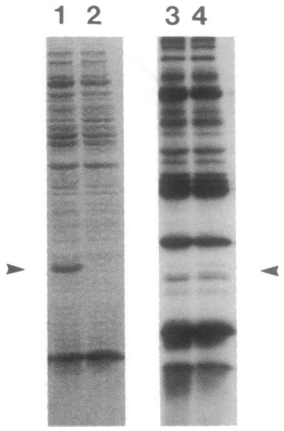

FIG. 5. Analysis of phosphorylation of RAP2B by PKA. Equal amounts (10 μl) of pTX-rap2b(+) extracts (lanes 1 and 3) or pTX-rap2b(−) extracts (lanes 2 and 4) were incubated with the catalytic subunit of PKA. The reaction products were analyzed by SDS/polyacrylamide gel electrophoresis, stained with Coomassie blue, and exposed to film. Lanes: 1 and 2, Coomassie stain profile; 3 and 4, autoradiograph. Arrowhead denotes the position of RAP2B in the gel. This experiment is representative of three others.

RAP2, we chose to call this cDNA and encoded protein RAP2B. Expression of the entire *RAP2B* sequence at high levels in *E. coli* demonstrated a protein of M_r 22,000 on SDS/polyacrylamide gels that bound monoclonal antibody M90 and GTP. The GTP binding was displaced by unlabeled GTP or GDP but was unaffected by unlabeled ATP. Moreover, incubation of the *E. coli* extracts with the catalytic subunit of PKA did not show any phosphorylation of the M_r 22,000 protein demonstrating that RAP2B was not a substrate for PKA, as has been observed for RAP1A and RAP1B.

Pizon *et al.* (14) initially isolated *RAP2* and *RAP1A* by screening a human cDNA library from Raji (human Burkitt lymphoma) cells at low stringency with the *Drosophila Dras3* gene. Their interest in the *Dras3* gene product and its potential similar human counterparts stemmed from the observation that amino acid 61 of the *Dras3* gene product is a threonine residue instead of the nontransforming glutamine in normal RAS. It had been shown by Der *et al.* (30) that almost every change at amino acid 61 in HRAS is associated with transformation; therefore, the presence of normal RAS-like proteins with a change at position 61 may be of interest. Since both RAP2 and RAP2B proteins contain a threonine residue at position 61, the intrinsic GTPase activity for these proteins may be lower, thereby allowing them to exist in the activated state a longer period of time than RAS p21.

Analysis of the DNA sequence of *RAP2B* indicated that the *RAP2B* gene should encode a protein of 183 amino acids with a deduced molecular weight of 20,434. This is similar to the M_r 22,000 protein observed in the *E. coli* lysates containing expressed RAP2B protein. Comparison of the deduced amino acid sequence of RAP2B with other small molecular weight proteins showed that it was most similar to RAP1A and RAP1B with 61% identity but was 46% homologous to KRAS. The regions of the highest homology of RAP2B and KRAS were amino acids 10–17, 55–62, 103–120, and 143–147; all of these regions contain residues involved in binding guanine nucleotides (31). Also conserved between KRAS and RAP2B are amino acids 32–42, which are implicated in the binding of the effector/GTPase-activating proteins (GAPs). Whether RAP2B interacts or competes with the same proteins as RAS p21 in this region is an area of active investigation.

RAP2B contains the canonical Cys-Ali-Ali-Xaa motif found in most of the small molecular weight GTP-binding proteins. This sequence has been implicated in the processing

of RAS p21 proteins for their ultimate insertion into the plasma membrane. Cysteine-180 would be the probable site of polyisoprenylation and cysteine-176 or -177 would be consistent with palmitoylation sequences (28). In fact, platelets that were labeled with [^{3}H]mevalonic acid showed incorporation of radiolabel in a M_r 21,000 protein that was immunoprecipitated by an antiserum raised against the RAP2B protein expressed in *E. coli*. Western blot analysis with the same antiserum showed that a M_r 21,000 protein was observed in the platelet membrane fraction and that a similar protein was immunoprecipitated from the particulate fraction of [^{35}S]methionine-labeled human erythroleukemia cells consistent with the posttranslational modification of RAP2B (D. A. Winegar, L. M. Molina y Vedia, and E.G.L., unpublished data). These data also support the presence of the *RAP2B* gene product in several platelets.

In summary, platelets contain low molecular weight RAS-like proteins including RAP1B, a M_r 22,000 protein that is translocated from the membrane to the cytosol after PKA phosphorylation (7, 12) and, as shown in this manuscript, RAP2B. The interactions that all of these proteins have with various receptors, GTPase-activating proteins, effectors, or any combination of these compounds as well as the ultimate effect on platelet function remains to be elucidated.

Note Added in Proof. The complete cDNA sequence of RAP2B will be published elsewhere (32).

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