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A Novel Mammalian Ras GTPase-Activating Protein Which Has Phospholipid-Binding and Btk Homology Regions

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We have previously purified a novel GTPase-activating protein (GAP) for Ras which is immunologically distinct from the known Ras GAPs, p120GAP and neurofibromin (M. Maekawa, S. Nakamura, and S. Hattori, J. Biol. Chem. 268:22948–22952, 1993). On the basis of the partial amino acid sequence, we have obtained a cDNA which encodes the novel Ras GAP. The predicted protein consists of 847 amino acids whose calculated molecular mass, 96,369 Da, is close to the apparent molecular mass of the novel Ras GAP, 100 kDa. The amino acid sequence shows a high degree of similarity to the entire sequence of the *Drosophila melanogaster* Gap1 gene. When the catalytic domain of the novel GAP was compared with that of *Drosophila* Gap1, p120GAP, and neurofibromin, the highest degree of similarity was again observed with Gap1. Thus, we designated this gene Gap1^m, a mammalian counterpart of the *Drosophila* Gap1 gene. Expression of Gap1^m was relatively high in brain, placenta, and kidney tissues, and it was expressed at low levels in other tissues. A recombinant protein consisting of glutathione-*S*-transferase and the GAP-related domain of Gap1^m stimulated GTPase of normal Ras but not that of Ras having valine at the 12th residue. Expression of the same region in *Saccharomyces cerevisiae* suppressed the *ira2*$^-$ phenotype. In addition to the GAP catalytic domain, Gap1^m has two domains with sequence closely related to those of the phospholipid-binding domain of synaptotagmin and a region with similarity to the unique domain of Btk tyrosine kinase. These results clearly show that Gap1^m is a novel Ras GAP molecule of mammalian cells.

It has been shown that the biological activity of Ras is controlled by GTP-GDP conversion (26, 34). The equilibrium between GTP- and GDP-bound states may be regulated by Ras guanine nucleotide-releasing factor and Ras GTPase-activating protein (GAP). Trahey and McCormick first described GAP activity which stimulates GTPase of the normal but not the oncogenic form of Ras (34). GAP was purified from bovine brains (6), and subsequently bovine and human GAP cDNAs were cloned (35, 36). GAP is a monomeric protein with a molecular mass of 116 kDa (p120GAP) (6, 35, 36).

Neurofibromin, a product of neurofibromatosis type I gene, has a domain whose sequence resembles that of the catalytic domain of p120GAP (40). This GAP-related domain (GRD) of neurofibromin (NF1-GRD) has been shown to possess GAP activity (1, 15, 39). *Saccharomyces cerevisiae IRA1* and *IRA2* and *Schizosaccharomyces pombe sar1* genes were isolated genetically as negative regulators of Ras (31, 33, 37). These genes also have a GRD. The functional interchangeability of both p120GAP and NF1 with the *IRA* gene has been demonstrated (32, 39). The *Drosophila* Gap1 gene has been isolated as a gene whose loss of function results in the oversignaling of Ras in the differentiation of photoreceptor cells (5). Although the Gap1 gene has not been characterized extensively, it has been considered a *Drosophila* counterpart of mammalian p120GAP, because Gap1 is the only GAP-related gene identified in *Drosophila melanogaster* at present.

Besides their function as a GAP, Ras GAPs interact with other important molecules. p120GAP has two Src homology regions (SH) 2, one SH3 region, and one pleckstrin homology (PH) region (11, 16, 19). Consistent with this feature is the fact that p120GAP binds to the activated epidermal growth factor and platelet-derived growth factor receptors (9, 10) and also to the tyrosine-phosphorylated proteins of 62 kDa (p62) and 190 kDa (p190) (10, 29, 38). Overexpression of the p120GAP SH regions in fibroblasts results in the promotion of growth (18) and the reduction of actin stress fibers (17). Neurofibromin binds to dimeric or polymerized tubulin (2).

We have purified a novel Ras GAP whose activity is neutralized neither by anti-p120GAP nor antineurofibromin (7, 14). Since its molecular mass, 100 kDa, is different from those of p120GAP and neurofibromin, we named this novel Ras GAP p100GAP^{Ras} (14). Although the immunogenicity and molecular weight of p100GAP differ from those of p120GAP and neurofibromin, its substrate specificity was comparable to theirs, in that p100GAP stimulates GTPase of only the normal Ras but not that of the oncogenic form of Ras or other low-molecular-weight GTP-binding proteins. The affinity of p100GAP for Ras was similar to that of neurofibromin and much higher than that of p120GAP (14).

In this study we have isolated cDNA which encodes p100GAP. The predicted amino acid sequence shows the most striking similarity to the entire sequence of the *Drosophila* Gap1 gene. Thus, we designated this gene Gap1^m, a mammalian counterpart of the Gap1 gene. Searching for homology to known protein sequences revealed that in addition to the GAP catalytic domain, Gap1^m has two putative phospholipid-binding domains and a region similar to the unique domain of Btk tyrosine kinase.

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MATERIALS AND METHODS

Isolation of Gap1^m cDNA. p100GAP was purified from rat brain tissue as described previously (14). After TSK-heparin high-performance liquid column chromatography, the sample, which contained about 3 μg of p100GAP, was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and the proteins were transferred to a polyvinylidene difluoride membrane. The immobilized p100GAP was reduced and S-carboxymethylated in situ prior to a sequential protease digestion by *Achromobacter* protease I (lysylendopeptidase), endoproteinase Asp-N, and trypsin (8). Peptides released from the membrane after each digestion were loaded onto a Wakosil-II AR reverse-phase high-performance liquid chromatography column (2.0 by 150 mm; Wako) preequilibrated with 0.05% trifluoroacetic acid. Peptides were eluted by 2 to 50% linear gradient of 2-propanol–acetonitrile (7:3 [vol/vol]) in 0.02% trifluoroacetic acid. Amino acid sequence analysis was performed with a gas-phase sequencer (model PSQ-2; Shimadzu).

PCR was performed by using degenerate oligonucleotides corresponding to the two peptide sequences, AP-6 and AP-2 (marked by boxes in Fig. 1A). Thirty cycles of the reaction, with one cycle consisting of 1.5 min at 94°C, 2 min at 54°C, and 3 min at 72°C were performed to amplify a rat adult brain cDNA library {Clontech RL1043b [oligo(dT) and randomly primed]}. The amplified DNA was used to screen the same library (2.0×10^6), which yielded three independent lambda phage clones with overlapping inserts (Gap1^m-4, Gap1^m-5, and Gap1^m-7).

For the isolation of 153-bp 5′-terminal sequence, 5′ extension was carried out as described by Frohman et al. (4), using a 5′ Amplifinder RACE kit supplied by Clontech. An oligonucleotide Gap1-GGC complementary to nucleotides 391 to 406 was used as a primer, and 2 μg of poly(A) RNA from 17-day-old embryonic rat brain tissue was employed as a template. After ligation of the anchor sequence attached to the kit, the product was amplified by PCR, using an oligonucleotide Gap1-GGG complementary to nucleotides 254 to 279 and an oligonucleotide complementary to the anchor sequence. The lambda phage inserts and the PCR product were cloned into pUC18 (42) for sequencing. The nucleotide sequence was determined as previously described (25) with a Pharmacia ALF sequencer.

Expression of Gap1^m-GRD in *Escherichia coli*. A *Bam*HI fragment of Gap1^m-5 (amino acid residues 306 to 847, nucleotides 970 to 2752) was ligated into the *Bam*HI site of pGEX-2T (Pharmacia) and was expressed in *E. coli* as a fusion protein with glutathione-*S*-transferase (GST). The recombinant protein was purified by glutathione-Sepharose (Pharmacia). GAP activity was measured by using Ras p21 proteins (27) as previously described (6), except that the incubation temperature was 33°C.

Suppression of heat sensitivity of *S. cerevisiae* carrying *ira2*$^-$ mutation by Gap1^m-GRD. *S. cerevisiae* KT27-2B (*MAT***a** *ura3 leu2 trp1 his3 ira2*$^-$) was used in this study. Transformants of various plasmids were grown in SDC medium containing 2% glucose, 0.67% yeast nitrogen base with no amino acids (Difco), 0.3% Casamino Acids (Difco), 20 mg of adenosine sulfate per liter, and 20 mg of L-tryptophan per liter. The same *Bam*HI fragment as described above was blunt ended and then cloned into the *Pvu*II site of the *S. cerevisiae* expression vector pKT10 (33) which contains a glyceraldehyde-3-phosphate dehydrogenase promoter. Construction of an NF1-GRD expression plasmid was described previously (39). Three independent transformants were selected, and their sensitivities to heat treatment were tested as described by Tanaka et al. (31).

Analysis of Gap1^m expression. Total RNAs from various adult rat tissues were subjected to reverse transcription-PCR analysis, employing the oligonucleotides corresponding to AP-6 and AP-2 as described above. The products were analyzed by 1.2% agarose gel electrophoresis.

Nucleotide sequence accession number. The GenBank accession number for Gap1^m cDNA sequence is D30734.

RESULTS

We have reported the purification of a novel mammalian Ras GAP, p100GAP, from rat brain tissue (14). To understand the biochemical properties and cell biological roles of p100GAP at the molecular level, we attempted to isolate its cDNA. The partial amino acid sequence was determined, which enabled us to design degenerate oligonucleotides to carry out PCR amplification of the adult rat brain cDNA library. Among the possible combinations two oligonucleotides corresponding to the two peptides (the boxed sequences in Fig. 1A) yielded the specific 520-bp fragment. Nucleotide sequencing of this fragment predicted that it could encode another peptide derived from p100GAP. Thus, this fragment was used to screen the same library. Three independent lambda phage clones were obtained from 2.0×10^6 clones. The 2.7-kb insert of Gap1^m-5 covers almost all the coding sequence. The 5′-terminal sequence (153 bp) was determined by sequencing the PCR-amplified 5′ extension product obtained by the method described in Materials and Methods.

The longest open reading frame of the cDNA could encode a protein consisted of 847 amino acids, and its calculated molecular mass, 96,369 Da, is close to the apparent molecular mass, 100 kDa (14). The flanking sequence of the initiation codon completely fits the rule proposed by Kozak (12). The termination codon TAG is present 21 bp upstream of the initiation codon. The predicted amino acid sequence is shown in Fig. 1A. Analysis of homology with protein sequences deposited in GenBank data base was carried out as described by Pearson and Lipman (21). The highest score was observed between the entire sequence of p100GAP and *Drosophila* Gap1 (39.3% identity [data not shown]). Thus, we named this gene Gap1^m to indicate that it is the mammalian counterpart of *Drosophila* Gap1. When the GRD of Gap1^m is compared with the corresponding regions of *Drosophila* Gap1, p120GAP, and neurofibromin, identities of 45.4, 25.7, and 26.5%, respectively, are found (Fig. 1B). Again, *Drosophila* Gap1 shows the highest similarity. Gap1^m-GRD also shows 24.9 and 24.5% identities to the GRDs of *IRA2* gene of *S. cerevisiae* and to the *sar1* gene of *S. pombe*, respectively (data not shown). In addition to the similarity among GRD sequences, both Gap1^m and p120GAP have a putative phospholipid-binding domain as described below. Flanking sequences of neurofibromin do not show significant similarity to Gap1^m.

To further confirm that Gap1^m has GAP activity, part of Gap1^m (*Bam*HI fragment of Gap1^m-5, amino acids 306 to 847) which contained the GRD sequence was expressed in *E. coli* as a GST fusion protein. The recombinant protein was purified by affinity chromatography on glutathione-Sepharose. GAP activity toward the normal but not the oncogenic Ras was clearly demonstrated (Fig. 2). This property is the same as that already reported for Gap1^m protein purified from rat brain tissue (14).

S. cerevisiae carrying *ira2*$^-$ lesions show hypersensitivity to heat treatment, possibly due to the constitutive activation of

A

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  1  MAAAAPAAAA LTEAPAVPGT AEPETGDEDS REVRVLQSLR GRIYEAKNLL
 51  PYLGPNKMRD CFCTINLDQE EVYRTQVVEK SLSPYFSEEF YFEIPRTFQY
101  LSFYVYDKNV LQRDLRIGKV AIKKEDLCSH SGKETWFSLQ PIDSNSEVQG
151  KVHLELKLNE LITENGTVCQ QLVVHIKACH GLPLINGQSC DPYATVSLVG
201  PSRNDQKKTK VKKKTSNPQF NEVFYFEVTR SSSYTRKSQF QVEEEDIEKL
251  EIRIDLWNNE NLVQDVFLGE IKVPVNVLRN DSSHQAWYLL QPRDNGNKSS
301  KPDDLGSLLL TLCYTEDYVL PSEYYGPLKA LLLKSPDVQP VSASAAYILG
351  EICRDQKDAV LPLVRLLLHH NKLVPFITAV ADLDLKDTQD ANAIFRGNSL
401  ATQCLTEMMK IVGGHYLKVT LKPVLDEICE SSKSCEIDPV KLKEGDNVES
451  NKENLYYYVD KVFSAIVGSS VSCPTVMCDI FYSLRQMAAK RFPNNPHVQY
501  SAVSSFVFLR FFAVAILSPH AFHLRPHYPD TQTVRTLTLI SKTIQIIGNW
551  GCQSRRKSRF KKSVMCEFLK MFQEERYFTD VKKFLDEISS TETKESSGTS
601  EPVHLKEGEM YKRAQGRTRI GKKNFKKRWF CLTSKELTYH KQQGKDAIYT
651  IPVKNILAVE KLEESSFNKK NMFQVIHTEK TLYIQANNCV EANEWIDMLC
701  RVSRCNHNRL SSFHPSAYLN GNWLCCQETS EGTPGCKPCT AGIPADIQID
751  IDEDRETERI YSVFTLSLLK LQKMEEACGS IAVYQGPQKE PGYSKFTIED
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B

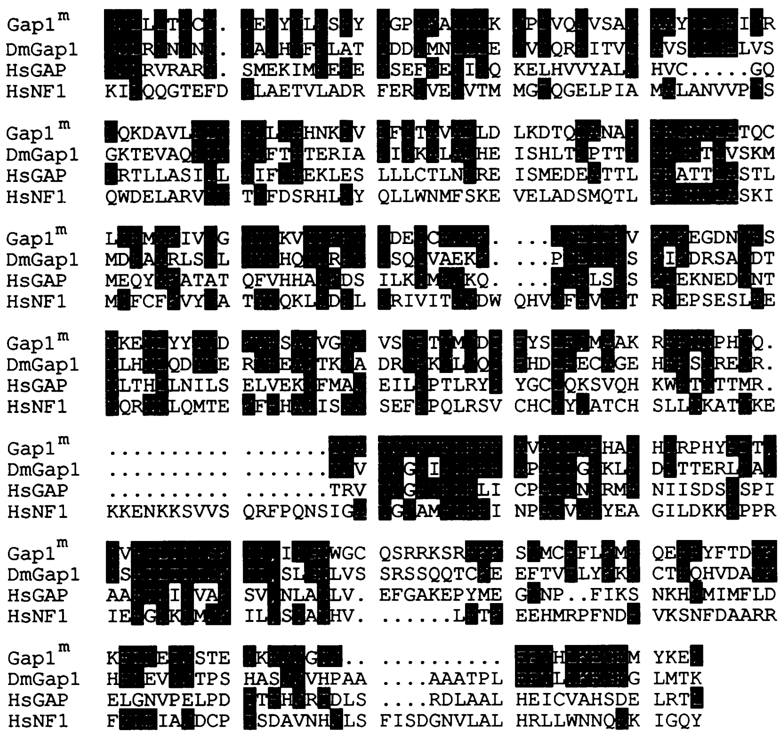

FIG. 1. Amino acid sequence of Gap1^{m} (A) and similarity among GRDs of Gap1^{m}, p120GAP, and neurofibromin (B). (A) The amino acid sequence of Gap1^{m} deduced from the nucleotide sequence of Gap1^{m} cDNA is shown. The sequences of the peptides derived from purified p100GAP are underlined. The two boxed sequences are those peptides whose degenerate oligonucleotides were used to carry out the PCR. (B) Sequence similarity among the GRDs of Gap1^{m} (residues 306 to 609), *Drosophila* Gap1 (DmGap1) (M86655; residues 455 to 771), human p120GAP (HsGAP) (P20936; residues 704 to 1012), and human neurofibromin (HsNF1) (M89914; residues 1185 to 1521). Amino acids that are identical in Gap1^{m} and other Ras GAPs are indicated by white lettering on a black background.

the RAS-cyclic AMP signaling pathway, since this phenotype is similar to that observed in those cells expressing the activated form of *RAS2* (33). Expression of the GRD of either p120GAP or neurofibromin complements the *ira2*$^{-}$ phenotype of *S. cerevisiae*, which shows that both mammalian Ras GAPs could function in *S. cerevisiae* as a negative regulator of RAS (32). The same fragment as was used in making the GST fusion protein was ligated into the *S. cerevisiae* expression vector pKT10 (33) and introduced into the *ira2*$^{-}$ strain. Three independent clones were tested for sensitivity to heat treatment at 57°C for 20 min (Fig. 3). The growth of *S. cerevisiae* carrying the control vector or antisense Gap1^{m} was hardly visible, while the growth of *S. cerevisiae* expressing Gap1^{m} or the GRD of neurofibromin was apparent, indicating that these cells survived during the heat treatment. This result clearly demonstrates that Gap1^{m} could function in *S. cerevisiae* as a regulator of RAS.

The tissue specificity of Gap1^{m} expression in adult rats was analyzed. Total RNA from each tissue was amplified by reverse transcription-PCR, and the products were analyzed by agarose gel electrophoresis (Fig. 4). The size of each product was exactly the same as predicted from the nucleotide sequence. Gap1^{m} expression is relatively high in brain, placenta, and kidney tissues. Low-level expression was observed in other

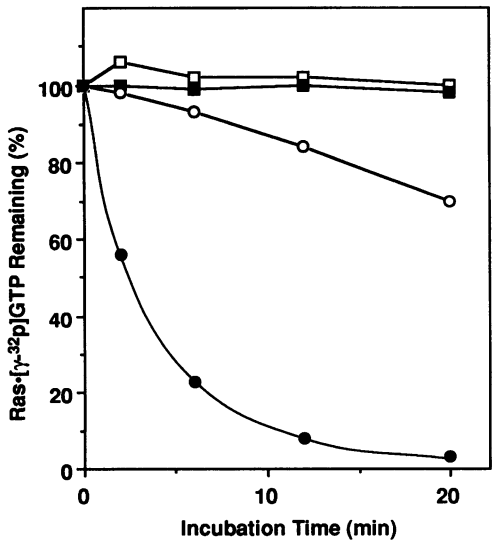

FIG. 2. GAP activity of GST fusion protein of Gap1^m. The *Bam*HI fragment of Gap1^m-5 (amino acid residues 306 to 847) was cloned into pGEX-2T (Pharmacia). Recombinant protein was affinity purified by using glutathione-Sepharose and then analyzed. The GTPase activities of wild-type Ras (circle) and Ras^{Val-12} (square) (50 ng each) were measured with (filled symbols) or without (open symbols) 140 ng of Gap1^m as previously described (6), except that the reaction was carried out at 33°C. The amount of Ras · [γ-^{32}P]GTP of samples kept on ice (3.5 to 4.0 pmol) was taken as 100%, and the relative values are shown.

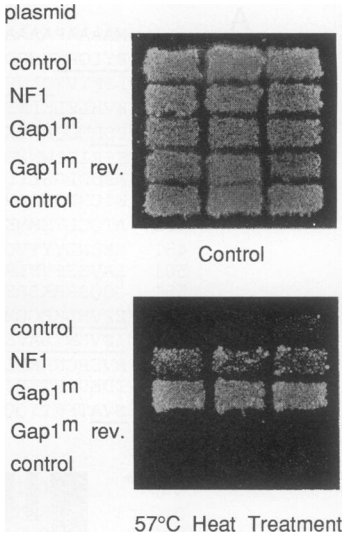

FIG. 3. Suppression of heat shock-sensitive phenotype of *ira2*$^-$ by the Gap1^m expression plasmid. pKT10 (control vector), pKP11 (expressing human NF1-GRD [NF1]) (39), and pKTGap1^m (expressing Gap1^m), pKTGap1^m · rev (expressing antisense Gap1^m [Gap1^m rev.]) were introduced into the *ira2*$^-$ strain KT27-2B. Three independent transformants carrying each plasmid were freshly grown at 30°C for 48 h and replica plated on a new plate. The plate was subjected to heat treatment at 57°C for 20 min and subsequently incubated at 30°C for 2 days. Heat shock-resistant cells survived and grew on the plate.

tissues. The intensities of reverse-transcribed cDNAs of the sample were quite similar (data not shown).

DISCUSSION

In this study we have presented evidence that there are at least three distinct Ras GAPs in mammalian cells: p120GAP, neurofibromin, and Gap1^m. Thus, it may be highly interesting to clarify their specific roles in the Ras signal transduction pathways. Since the predicted amino acid sequence has the highest similarity to almost the entire region of *Drosophila* Gap1, it may be reasonable to consider that our isolate may be a mammalian counterpart.

In a previous study (14), we reported that the affinity of Gap1^m to Ras · GTP-γS is similar to that of neurofibromin and is much higher than that of p120GAP. However, comparison of the catalytic domains of these Ras GAPs reveals that they resemble each other to a similar extent. Wang et al. pointed out a motif FLR(XXX)P[AV](XXX)P, which is commonly observed in all known Ras GAPs, where X is any amino acid and [AV] are the preferred amino acids (37). Gap1^m has valine instead of proline as the seventh amino acid.

Although the activities of three Ras GAP molecules are high in brain tissue, their intracellular distribution may be different. p120GAP is predominantly located in the cytoplasmic fraction, which is in contrast with neurofibromin, the major activity of which resides in the particulate fraction (7). Gap1^m activity is observed in both the soluble and particulate fractions (7, 14). These three GAPs contribute almost equally to the total GAP activity of brain tissue (7). In situ hybridization of brain tissue at the anatomical level may reveal tissue specificity of expression of these GAPs.

Despite having the same function as Ras GAP, these GAPs may have other specific roles, because their structural features are quite different from one another. p120GAP has two SH2 regions, one SH3 region, and one PH region in addition to GRD. Overexpression of the SH regions in fibroblasts results in the promotion of growth (18) and in the reduction of actin stress fibers (17). These SH regions interact with other molecules including p62 and p190. Neurofibromin is reported to interact with dimeric or polymerized tubulin (2). Flanking sequences of the GRD of neurofibromin show sequence similarity to *IRA* genes of *S. cerevisiae* but not to other known proteins.

These findings prompted us to look for the possible domain structure of Gap1^m in more detail. Figure 5A schematically illustrates that Gap1^m has two repeated units of the sequence with similarity to the C2 domain of protein kinase C at the amino terminus adjacent to GRD and also has the Btk homology region at the carboxy-terminal portion. Gap1^m does not have an SH2 or SH3 region.

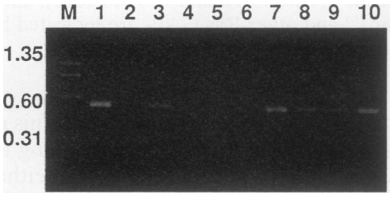

FIG. 4. Tissue specificity of Gap1^m expression. Total RNA (5 μg) from various tissues of adult rats was subjected to reverse transcription-PCR analysis by using the oligonucleotides AP-6 and AP-2. The product was analyzed by electrophoresis on 1.2% agarose. The positions (in kilobase pairs) of the size markers (M: *Hae*III digest of φX174 DNA) are shown to the left of the gel. The samples are from brain (lane 1), lung (lane 2), heart (lane 3), liver (lane 4), spleen (lane 5), adrenal gland (lane 6), kidney (lane 7), prostate gland (lane 8), testis (lane 9), and placenta (lane 10) tissue.

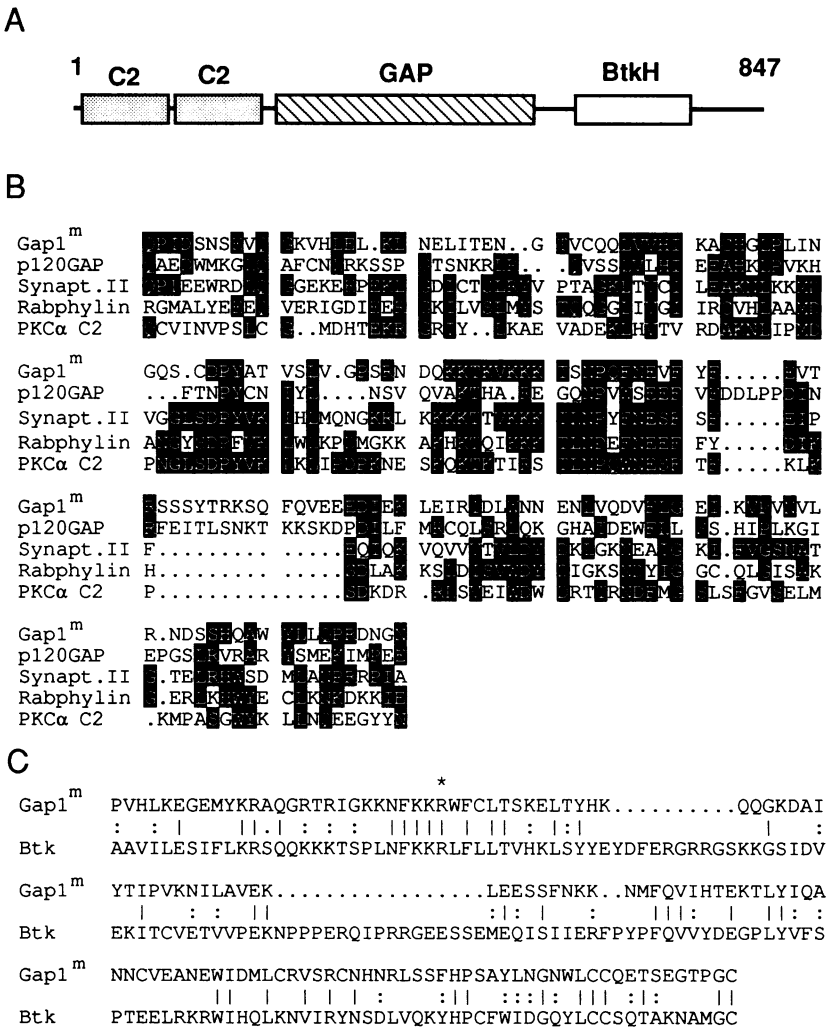

FIG. 5. Domain structure of Gap1^m. (A) Schematic illustration of Gap1^m. C2, GAP, and BtkH stand for the domains with sequence similarity to the C2 domain of protein kinase C, GRD, and Btk tyrosine kinase, respectively. The numbers are amino acids. (B) Sequence similarity among Gap1^m (amino acid residues 140 to 297), human p120GAP (residues 564 to 721), rat synaptotagmin II (Synapt.II) (GenBank accession number M64488; residues 255 to 403), bovine rabphylin (D13613; residues 542 to 690), and rat protein kinase C (PKCα C2) (X07286; residues 142 to 287). Amino acids that are identical in at least two sequences are indicated by white lettering on a black background. (C) Similarity between Gap1^m (amino acids 602 to 736) to the amino-terminal unique domain of human Btk (X58957; amino acids 2 to 165) is illustrated. Identical amino acids are shown by vertical bars, and conserved amino acid substitutions are indicated by colons. Dashes indicate gaps to maximize alignment. Conserved amino acid substitutions are grouped as follows: C; S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; and F, Y, and W. The 28th arginine residue which is replaced by cysteine in Xid mice (24) is marked by an asterisk.

Figure 5B shows the sequence comparison between Gap1^m and the phospholipid-binding proteins including synaptotagmin (22), rabphylin (30), and protein kinase C (20). This region of protein kinase C is called the C2 domain and binds Ca^{2+} in a phospholipid-dependent manner (13). It has also been shown that these regions of synaptotagmin and rabphylin have Ca^{2+}-dependent binding activity for phospholipids (23, 41). Both synaptotagmin and rabphylin have two repeated C2 domains. Gap1^m also has two C2 units with comparable similarity. In Fig. 5B, the C2 unit at the carboxy-terminal side is shown. Although the extent is less, p120GAP also has one domain similar to these proteins between its pleckstrin homology region and GRD (3). Consistent with this feature is the fact that the GAP activity of p120GAP is inhibited by certain species of phospholipids (28). We also observe that these phospholipids modulate activities of both p120GAP and Gap1^m (13a). The interaction with phospholipids may be important for the regulation of activity and the translocation of p120GAP. It is thus quite interesting to examine whether Gap1^m becomes translocated upon stimulation of the cells just as observed with p120GAP. *Drosophila* Gap1 also has this putative phospholipid-binding domain (data not shown).

Another interesting feature of Gap1^m is that the unique region of Btk tyrosine kinase family and Gap1^m both have domains with sequence similarity. This similarity is pointed out for Btk and *Drosophila* Gap1 by Rawlings et al. (24). Gap1^m resembles Btk more than *Drosophila* Gap1 does. In this region, the 28th arginine residue of Btk is replaced by cysteine by a point mutation in Xid mice (24), which may be the cause of agammaglobulinemia resulting from immature B cell lineages. Recently this domain of Btk has been considered a pleckstrin domain (19). However, the degree of similarity of Gap1^m to

Btk is much higher, and the region of similarity is much wider than those between Gap1^m and pleckstrin domains of other proteins.

The unique region of Btk is located outside the conserved tyrosine kinase domain. However, a mutation in this region in Xid mice impairs the Btk function, despite the fact that the mutant Btk protein still has normal kinase activity. One explanation for this dysfunction is that the interaction of Btk to a factor through this region may be important for signaling and that the mutation abrogates or decreases the interaction. In this context, it is interesting to speculate that these representative domains of Gap1^m and Btk family may bind to a common protein. A putative protein with unknown function whose sequence is very similar to the carboxy-terminal portion of Gap1^m is deposited in GenBank (accession number A09787). This sequence also shows homology to Btk (data not shown).

Our study has revealed a highly unique structure of Gap1^m, which has domains of phospholipid/Ca^{2+} binding, Ras GAP, and Btk homology. These features of Gap1^m are also conserved in *Drosophila* Gap1, which suggests the biologically important functions of these domains. Binding of Ras to Gap1^m might modulate the interaction of Gap1^m with phospholipid/Ca^{2+} or affect the binding of a putative protein to Btk homology region. The reverse is possible, in that binding of a putative protein and/or phospholipid/Ca^{2+} might affect GAP activity toward Ras. In either case, Gap1^m may have quite important roles in Ras signal transduction pathways.

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