Identification of AF-6 and Canoe as Putative Targets for Ras*

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Ras (Ha-Ras, Ki-Ras, N-Ras) is implicated in the regulation of various cell functions such as gene expression and cell proliferation downstream from specific extracellular signals. Here, we partially purified a Ras-interacting protein with molecular mass of about 180 kDa (p180) from bovine brain membrane extract by glutathione S-transferase (GST)-Ha-Ras affinity column chromatography. This protein bound to the GTP\*S (guanosine 5'-[3-O-thio]triphosphate, a nonhydrolyzable GTP analog)-GST-Ha-Ras affinity column but not to those containing GDP-GST-Ha-Ras or GTP\*S-GST-Ha-Ras with a mutation in the effector domain (Ha-Ras\*39). The amino acid sequences of the peptides derived from p180 were almost identical to those of human AF-6 that is identified as the fusion partner of the ALL-1 protein. The ALL-1/AF-6 chimeric protein is the critical product of the t(6;11) abnormality associated with acute human leukemia. AF-6 has a GLGF/Dlg homology repeat (DHR) motif and shows a high degree of sequence similarity with Drosophila Canoe, which is assumed to function downstream from Notch in a common developmental pathway. The recombinant N-terminal domain of AF-6 and Canoe specifically interacted with GTP\*S-GST-Ha-Ras. The known Ras target c-Raf-1 inhibited the interaction of AF-6 with GTP\*S-GST-Ha-Ras. These results indicate that AF-6 and Canoe are putative targets for Ras.

General Purpose

Ras (H-a-Ras, K-i-Ras, N-Ras) is a signal-transducing guanine nucleotide-binding protein for tyrosine kinase-type receptors such as epidermal growth factor receptors and the Src family, leading to a mitogenic response and differentiation (for reviews, see Refs. 1 and 2). Ras has GDP-bound inactive and GTP-bound active forms, the latter of which makes physical contact with targets. Intensive investigations revealed that the Raf kinase family, consisting of c-Raf-1 (for reviews, see Refs. 3 and 4), A-Raf (5), and B-Raf (6-9), is one of the direct targets for Ras. The activated Raf phosphorylates MAPK kinase and activates it. Consequently, the activated MAP kinase activates MAP kinase, leading to the expression of certain genes such as c-fos (for reviews, see Refs. 10 and 11). Several molecules interacting with activated Ras in addition to Raf have been identified in mammals. These include phosphatidylinositol-3-OH kinase (12), Raf GDS (13, 14), and Rin1 (15). On the basis of these observations, a variety of Ras targets may account for the pleotropic functions of Ras. To understand the molecular mechanism of pleiotropic functions of Ras, it is essential to identify novel targets for Ras.

In the present study, we discovered and partially purified another putative target for Ras with a molecular mass of about 180 kDa (p180) by use of GST-Ha-Ras affinity column chromatography and identified it as AF-6 (16), whose structure resembles that of Drosophila Canoe, which is involved in the Notch signaling pathway (17).

EXPERIMENTAL PROCEDURES

Materials and Chemicals—All materials used in the nucleic acid study were purchased from Takara Shuzo Co. Ltd. (Kyoto, J apan). Expression plasmids, pGEX, pMal-c2, and pRSET were obtained from Pharmacia Biotech (Tokyo, J apan), New England Biolabs Inc. (Beverly, MA), and Invitrogen Corp. (San Diego, CA), respectively. Other materials and chemicals were obtained from commercial sources. [35S]GTP\*S and [35S]methionine were purchased from DuPont NEN. A rabbit polyclonal antibody against a 16-mer peptide corresponding to 561–576 aa of human AF-6 (RVEQPDYRQESRTQ) was generated and purified.

Plasmid Construction—Plasmids, pGEX-Ha-Ras, pGEX-R-Ras, pGEX-Raf, and pGEX-RhoA were constructed as described previously (18). To obtain the in vitro translated N-terminal domain of AF-6 and Canoe, pRSET-AF-6 (36–848 aa), pRSET-AF-6 (36–206 aa), and pRSET-Canoe (1–217 aa) were constructed as follows. The 2.4-kilobase cDNA fragment encoding AF-6 (36–848 aa) was amplified by polymerase chain reaction from human brain Quick clone cDNA (Clontech Laboratories Inc., Palo Alto, CA). For the shorter N-terminal domain of AF-6, the 0.51-kilobase cDNA fragment encoding AF-6 (36–206 aa) was amplified by polymerase chain reaction from human brain Quick clone cDNA (Clontech Laboratories Inc., Palo Alto, CA). For the shorter N-terminal domain of AF-6 and Canoe specifically encoding Canoe (1–217 aa) were amplified by polymerase chain reaction from the AF-6 cDNA clone K12 (16) and Canoe cDNA clone in pBluescript-SK (17), respectively. These cDNA fragments, having an artificial termination codon and also artificial KpnI sites at both terminals, were cloned into the KpnI site of pRSET. To produce the shorter N-terminal domain of AF-6 and N-terminal domain of c-Raf-1 as MBP fusion proteins, the cDNA fragments encoding AF-6 (36–206 aa) and c-Raf-1 (1–149 aa) were subcloned into the KpnI site of pMal-c2-KpnI in which an additional KpnI site was introduced adjacent to the BamHI site. Preparation of Bovine Brain Membrane Extract—The homogenate of bovine brain gray matter, 190 g, was prepared and centrifuged at 20,000 × g for 30 min at 4°C as described (19). The precipitate was suspended into 360 ml of homogenizing buffer (25 mM Tris/HCl at pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl2, 10 μM (p-amidino- phenyl)-methanesulfonfyl fluoride, 1 mg/liter leupeptin, 10% sucrose) to prepare the crude membrane fraction (19). The proteins in this fraction were extracted by addition of an equal volume of homogenizing buffer

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‡ The abbreviations used are: MAP, mitogen-activated protein; Ral GDS, Ral guanine nucleotide dissociation stimulator; GST, glutathione S-transferase; AF-6, ALL-1 fusion partner from chromosome 6; ALL, acute lymphoblastic leukemia; GTP\*S, guanosine 5’-3-O-thiophosphate; aa, amino acids; MBP, maltose-binding protein; G proteins, GTP-binding proteins; PAGE, polyacrylamide gel electrophoresis.
methionine were mixed with glutathione-Sepharose 4B beads containing either GST, GDP-GST-Ha-Ras, GTPyS-GST-Ha-Ras, or GTPyS-GST-Ha-RasA38. Bound proteins were coeluted with the respective GST-fusion proteins by addition of glutathione. Aliquots (40 μl each) from the glutathione eluates were resolved by SDS-PAGE followed by silver staining. Lane 1, GST; lane 2, GST-GST-Ha-Ras; lane 3, GTPyS-GST-Ha-Ras; lane 4, GTPyS-GST-Ha-RasA38. The arrow and arrowhead denote the positions of p180 and p195, respectively. The results are representative of three independent experiments.

Brain membrane extract was loaded onto GST-Ha-Ras affinity column chromatography as described above. Neither p180 nor p195 was eluted from the GST-RalA or GST-RhoA affinity column (data not shown). Neither p180 nor p195 was eluted from the GTPyS-RalA or GST-RhoA affinity column (data not shown). Neither p180 nor p195 was eluted from the GST-RalA or GST-RhoA affinity column (data not shown).

To identify the GTPyS-Ha-Ras-interacting molecule, p180 was subjected to amino acid sequencing as described under “Experimental Procedures.” Six peptide sequences derived from p180 were determined. These were 1) STATQDVLE; 2) DMPETSFTR; 3) LLYLVELSPDG; 4) PGIVQETTFDLG; 5) DMPETSFTR; 3) LPYLVELSPDG; 4) PGIVQETTFDLG; 5) YAPDDIPNINS; and 6) LLLEWQFQK. All six peptide sequences were almost identical to the deduced amino acid sequence of human AF-6, which is the fusion partner of the ALL-1 protein (16). The ALL-1/AF-6 chimeric protein is the critical product of the (6;11) abnormality associated with some human leukemias. Furthermore, p180 was recognized by the antibody raised against human AF-6 (Fig. 2). The calculated molecular mass of human AF-6 is 181,777 Da, which is close to the apparent molecular mass of p180 estimated by SDS-PAGE. We therefore concluded that p180 is the bovine counterpart of human AF-6 and hereafter referred to it as AF-6. Since this antibody cross-reacted with p195 weakly (data not shown), p195 may be an isoform or an alternatively spliced form of AF-6.

To address whether or not recombinant AF-6 interacts with GTPyS-Ha-Ras, GST-small G proteins immobilized on beads were mixed with the in vitro translated N-terminal domain of AF-6 (36–848 aa), and interacting proteins were coeluted with GST-small G proteins by addition of glutathione. The incubation mixture was subjected to amino acid sequencing as described above.

Interaction of In Vitro Translated AF-6, Canoe, and Bacterial AF-6 with GST-Small G Proteins—In vitro translation of prSET-AF-6 (36–848 aa), prSET-AF-6 (36–206 aa), and prSET-Canoe (L-217 aa) were performed as described (19). In vitro translated products labeled with [35S]methionine were mixed with glutathione-Sepharose 4B beads containing the respective GST-small G proteins loaded with guanine nucleotides (19). The bound proteins were then coeluted with GST-small G proteins by addition of glutathione. The eluates were resolved by SDS-PAGE and vacuum-dried followed by autoradiography. The shorter N-terminal domain of AF-6 (36–206 aa) was expressed as an MBP fusion from the pRSET-AF-6 plasmid (New England BioLabs). MBP-AF-6 (0.15 nmol) was subjected to the GST-small G protein affinity column chromatography as described above. For competition assay with c-Raf-1, 1.5 nmol of MBP-c-Raf-1 was simultaneously added to the incubation mixture.

RESULTS AND DISCUSSION

To detect molecules interacting with Ha-Ras, the bovine brain membrane extract was loaded onto GST-Ha-Ras-affinity columns. The proteins bound to the affinity columns were coeluted with GST-Ha-Ras by addition of glutathione. Proteins with a molecular mass of about 180 kDa (p180) and 195 kDa (p195) were detected in the glutathione eluate from the GTPyS-GST-Ha-Ras affinity column but not from those containing GST or GDP-GST-Ha-Ras (Fig. 1). Neither p180 nor p195 was detected in the eluate of the affinity column for GTPyS-GST-Ha-RasA38, which has a mutation in the effector-interacting domain (1, 2). We further confirmed the specificity of the interaction by affinity column chromatography using GST-R-Ras, GST-RalA, and GST-RhoA. Less p180 and p195 were eluted from the GTPyS-GST-R-Ras affinity column but not from the GDP-GST-R-Ras affinity column (data not shown). Neither p180 nor p195 was eluted from the GST-RalA or GST-RhoA affinity column (data not shown).

To identify the GTPyS-Ha-Ras-interacting molecule, p180 was subjected to amino acid sequencing as described under “Experimental Procedures.” Six peptide sequences derived from p180 were determined. These were 1) STATQDVLE; 2) DMPETSFTR; 3) LLYLVELSPDG; 4) PGIVQETTFDLG; 5) YAPDDIPNINS; and 6) LLLEWQFQK. All six peptide sequences were almost identical to the deduced amino acid sequence of human AF-6, which is the fusion partner of the ALL-1 protein (16). The ALL-1/AF-6 chimeric protein is the critical product of the (6;11) abnormality associated with some human leukemias. Furthermore, p180 was recognized by the antibody raised against human AF-6 (Fig. 2). The calculated molecular mass of human AF-6 is 181,777 Da, which is close to the apparent molecular mass of p180 estimated by SDS-PAGE. We therefore concluded that p180 is the bovine counterpart of human AF-6 and hereafter referred to it as AF-6. Since this antibody cross-reacted with p195 weakly (data not shown), p195 may be an isoform or an alternatively spliced form of AF-6.

To address whether or not recombinant AF-6 interacts with GTPyS-Ha-Ras, GST-small G proteins immobilized on beads were mixed with the in vitro translated N-terminal domain of AF-6 (36–848 aa), and interacting proteins were coeluted with GST-small G proteins by addition of glutathione. The in vitro translated AF-6 (36–848 aa) was coeluted with GTPyS-GST-Ha-Ras but weakly with GDP-GST-Ha-Ras, GTPyS-GST-Ha-RasA38, GST-R-Ras, GST-RalA, and GST-RhoA (Fig. 3a). The band with GTPyS-GST-Ha-RasA38 was very faint (lane 5), and the bands with GDP-GST-RalA and GTPyS-GST-RalA (lanes 8 and 9) were a little bit stronger than those with GDP-GST-R-Ras and GTPyS-GST-R-Ras (lanes 6 and 7). The weak bands detected in the eluates other than that from GTPyS-GST-Ha-Ras may result from the weak interaction of AF-6 with the respective small G proteins. Although some AF-6 in the membrane extract was slightly retained on the GTPyS-GST-R-Ras affinity column (data not shown), the in vitro translated AF-6 was not. This may be due to the lower affinity of AF-6 for GTPyS-GST-R-Ras than that for GTPyS-GST-Ha-Ras. To determine the Ras-interacting domain of AF-6 more accurately, a similar experiment was performed using the shorter N-terminal domain of AF-6 (36–206 aa). A similar retention was observed when the shorter N-terminal domain of AF-6 (36–206 aa) was employed (Fig. 3b).

A homology search of the GenBank protein data base revealed a high degree of sequence similarity of AF-6 with Dro sophila Canoe (Fig. 4), which is assumed to function down-
The apparent $K_d$ values for MBP-AF-6 and MBP-c-Raf-1 were estimated to be about 250 and 200 nM, respectively, under the conditions (data not shown). Since c-Raf-1 interacts with activated Ras via the effector domain (1, 2), we examined whether or not c-Raf-1 competes with AF-6 for interaction with activated Ha-Ras. An excess amount of MBP-c-Raf-1 inhibited the interaction of the MBP-AF-6 with GTP$_7$S-GST-Ha-Ras (Fig. 3c).

In this study, we purified a Ras-interacting protein (p180) from a bovine brain membrane extract. We identified it as AF-6, which has a GLGF/DHR motif and shows a high degree of sequence similarity with Drosophila Canoe (16, 17). The recombinant AF-6 and Canoe specifically interacted with activated Ha-Ras. Furthermore, c-Raf-1 inhibited the interaction of AF-6 with activated Ha-Ras. These results indicate that AF-6 and Canoe serve as putative targets for Ras.

We showed that activated Ras interacted with the N-terminal domains of AF-6 and Canoe. These domains show a high degree of sequence similarity to each other, indicating that this unique domain confers specificity for the GTP-Ras complex. The direct interaction of c-Raf-1, A-Raf, B-Raf, phosphatidylinositol-3-OH kinase, Ral GDS, and Rin1 with activated Ras has been demonstrated (3–9, 12–15). The Ras-interacting domains of these proteins have been determined. There is no obvious homology among Ras-interacting interfaces of c-Raf-1, phosphatidylinositol-3-OH kinase, Ral GDS, Rin1, and AF-6/Canoe, indicating that activated Ras can recognize a variety of target interfaces. This diversity of Ras-interacting interfaces may allow a range of downstream pathways from Ras to induce appropriate cellular responses to extracellular signals.

AF-6 and Canoe are homologous to each other and share a common domain organization (Fig. 4) (16, 17). The most highly conserved region among them is a GLGF/DHR motif, which is found in a number of other proteins including Drosophila discs-large tumor suppressor gene product (Dig) (24), disheveled gene product (25, 26), an intracellular protein-tyrosine phosphatase (PTP-mer) (27), postsynaptic density protein 95 (PSD-95) (28), and a tight junction-associated protein ZO-1 (29, 30). The GLGF/DHR motif is thought to function to localize them at the specialized sites of cell-cell contact by forming a complex with specific proteins such as protein 4.1 homologues (31). The structural feature of AF-6 and Canoe suggests that they locate at the junction of plasma membrane and cytoskeleton, where they may regulate signal transduction and cytoskeleton.

The N terminus of AF-6 flanked by the GLGF/DHR motif also shares a high homology with that of Canoe, to both of which activated Ras binds specifically. Canoe has been postulated to function downstream from Notch and to mediate interactions between the Notch cascade and other signaling pathways (17). Although AF-6 function remains obscure, the similar structural feature and property of AF-6 and Canoe suggest that the AF-6/Canoe family may serve as an intracellular signaling component controlled by two distinct signaling pathways such as Ras and Notch. Our preliminary experiments suggest that Canoe is genetically linked to Ras1 in Drosophila eye development. Further studies are required to understand the roles of AF-6/Canoe family in signal transduction.

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