Mitogen-activated Protein Kinase Kinase Kinase 4 as a Putative Effector of Rap2 to Activate the c-Jun N-terminal Kinase*

Received for publication, December 12, 2003, and in revised form, February 11, 2004
Published, JBC Papers in Press, February 13, 2004, DOI 10.1074/jbc.C300542200

Noriko Machida‡, Masato Umikawa‡,
Kimiko Takei‡, Nariko Sakima‡,
Bat-Erdene Myagmar‡, Kiyohito Taira‡,
Hiroshi Uezato‡, Yoshhide Ogawa‡, and Ken-ichi Kariya‡

From the §Division of Cell Biology, Graduate School of Medicine, the Departments of §Dermatology and §Urology, Faculty of Medicine, University of the Ryukus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan.

Little is known about the specific signaling roles of Rap2, a Ras family small GTP-binding protein. In a search for novel Rap2-interacting proteins by the yeast two-hybrid system, we isolated isoform 3 of the human mitogen-activated protein kinase kinase kinase 4 (MAP4K4), a previously described but uncharacterized isoform. Other isoforms of MAP4K4 in humans and mice are known as hematopoietic progenitor kinase (HPK)/germlinal center kinase (GCK)-like kinase and Nck-interacting kinase, respectively. MAP4K4 belongs to the STE20 group of protein kinases and regulates c-Jun N-terminal kinase (JNK). MAP4K4 interacted with Rap2 through its C-terminal citron homology domain but did not interact with Rap1 or Ras. Interaction with Rap2 required the intact effector region of Rap2. MAP4K4 interacted preferentially with GTP-bound Rap2 over GDP-bound Rap2 in vitro. In cultured cells, MAP4K4 co-localized with Rap2, while a mutant MAP4K4 lacking the citron homology domain failed to do so. Furthermore, Rap2 enhanced MAP4K4-induced activation of JNK. These results suggest that MAP4K4 is a putative effector of Rap2 mediating the activation of JNK by Rap2.

Rap2 belongs to the Ras family of small GTP-binding proteins. The mammalian Ras family consists of Ras proteins (Ha-Ras, Ki-Ras, and N-Ras), Rap proteins (Rap1A, Rap1B, Rap2A, and Rap2B), R-Ras, R-Ras2/TC21, R-Ras3/M-Ras, Raf proteins (RafA and RafB), Rheb, Rin, and Rit (for a review, see Ref. 1). Orthologs of both Rap1 and Rap2 are present in the nematode Caenorhabditis elegans, suggesting that Rap1 and Rap2 play important and distinct roles. However, little is known about the specific roles of Rap2 in cell signaling.

The Ras family proteins regulate many cellular processes including proliferation, differentiation, and apoptosis (1). In regulating these processes, they serve as molecular switches by cycling between GTP-bound active and GDP-bound inactive forms. The GTP-bound forms interact physically with downstream effectors. Interactions of Ras family proteins with effectors are mediated by effector regions (in the case of Ras, amino acids 32–40). The effector regions of the Ras family proteins also play critical roles in differential recognition of effectors.

An effector region identical to that of Ras is present in Rap1. Rap1 interacts with most of the effectors of Ras, including Raf-1 and B-raf, both of which are upstream of the “classical” mitogen-activated protein kinase (MAPK), the extracellular signal-regulated kinase. Although Rap1 interacts with both Raf-1 and B-raf, it activates only B-raf (1). Rap1 does not have its specific effector, like the other Ras family members sharing the identical effector region.

The effector region of Rap2 differs from those of Ras and Rap1 by a single amino acid: amino acid 39 in Rap2 is Phe, while in Ras and Rap1 it is Ser. This difference could confer on Rap2 an ability to interact with its specific effectors and play signaling roles distinct from those of Rap1. Here, we report the identification of a novel Rap2-interacting protein, human MAP kinase kinase kinase kinase (MAP4K4) isoform 3, a previously described but uncharacterized isoform of MAP4K4 (2–4).

MAP4K4 exhibits structural homology to hematopoietic progenitor kinase (HPK) and germinal center kinase (GCK) (for a review, see Ref. 5). Thus, human MAP4K4 was designated HPK/GCK-like kinase (HGK) (2). Mouse MAP4K4 was discovered as a kinase that interacts with Nck, an adaptor protein composed of one Src homology (SH) 2 and three SH3 domains, and hence designated Nck-interacting kinase (NIK) (3) (Fig. 1A). MAP4K4 belongs to a large group of kinases related to Saccharomyces cerevisiae MAP4K, STE20. The STE20 group is divided into two families (for a review, see Ref. 6). One is the p21-activated kinase family, whose members are activated by the Rho family small GTP-binding proteins Cdc42 and Rac. The other is the GCK family, which consists of eight subfamilies. GCK and HPK, also known as MAP4K2 and MAP4K1, respectively, belong to the GCK-I subfamily; MAP4K4 belongs to the GCK-IV subfamily. Members of both GCK-I and -IV subfamilies are upstream of c-Jun N-terminal kinase (JNK), a “stress-activated” MAPK. In the present study, we show that Rap2 enhances activation of JNK by MAP4K4. We postulate that Rap2 plays signaling roles distinct from those of Rap1 by regulating JNK.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAP4K, MAP kinase kinase kinase kinase; HPK, hematopoietic progenitor kinase; GCK, germinal center kinase; HGK, HPK/GCK-like kinase; SH, Src homology; NIK, Nck-interacting kinase; CNH, citron homology; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; HA, hemagglutinin; GTPγS, guanosine 5′-O-(thiotriphosphate).
Two-hybrid Screening—A cDNA fragment encoding amino acids 1–168 of human Rap2A was subcloned into plasmid pBTM116 to yield pBTM116-Rap2A. pBTM116-Rap2A and the human fetal brain cDNA library cloned in pACT2 (Clontech, Palo Alto, CA) were co-transformed into the S. cerevisiae L40 reporter strain. Approximately 4.7 × 10⁶ co-transformants were examined for HIS3 and β-galactosidase expression as described previously (7).

In Vitro Binding Assay—Full-length Rap2A (amino acids 1–183) and Ha-Ras were expressed in Sf9 insect cells as fusion proteins with glutathione S-transferase (GST) using the baculovirus expression system as described previously (7). GST-Rap2A or GST-Ha-Ras was immobilized on glutathione-Sepharose resin (Amersham Biosciences). The coding sequence for full-length MAP4K4 isoform 3 (amino acids 1–1212) (Fig. 1A) was generated by using a PCR-amplified cDNA sequence encoding amino acids 1–208 (common to all isoforms) and a cDNA clone encoding amino acids 38–1212 of this isoform (KIAA0687), provided by Dr. Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). HEK293T cells were transfected with pCIneo-HA-MAP4K4 or pCIneo-HA-MAP4K4ΔCNH expressing HA-tagged full-length MAP4K4 or its deletion mutant lacking the CNH domain using Polyfect reagent (Qiagen). Cells were harvested, homogenized in buffer A (20 mM Tris/HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂, 0.1% Nonidet P-40) containing protease inhibitors (Roche Applied Science), and centrifuged at 100,000 × g for 1 h. The supernatant (the soluble fraction) was incubated with the glutathione-Sepharose resin carrying immobilized GST-Rap2A or GST-Ha-Ras preloaded with GTP·S or GDP as described previously (7).

Fluorescence Microscopy—NIH3T3 cells were fixed with formaldehyde and processed for immunofluorescence confocal microscopy (LSM 5 PASCAL, Carl Zeiss) with monoclonal anti-Myc antibody (9E10; Cell Signaling Technology, Beverly, MA) and polyclonal anti-HA antibody (sc-805; Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (8).

JNK Activation Assay—pFLAG-CMV-2JNK2 and pGEX2T-c-Jun(1–79) were kindly provided by Dr. Katsui Yoshioka (Kanazawa University, Ishikawa, Japan) (9). 293T cells were homogenized in buffer A containing protease inhibitors, and homogenates were subjected to Western immunoblotting with polyclonal anti-phospho-JNK (Thr-183/Tyr-185) antibody (number 9251; Cell Signaling Technology). The homogenates were also subjected to an immunocomplex kinase assay as described previously (8) except that FLAG-JNK2 was immunoprecipitated with anti-FLAG M2 resin (Sigma), and the levels of phosphorylation of GST-c-Jun (1–79) were assessed by Western immunoblotting with polyclonal anti-phospho-c-Jun (Ser-63) antibody (number 9246; Cell Signaling Technology).

RESULTS

To identify a specific effector of Rap2, we carried out a yeast two-hybrid screening for Rap2-interacting proteins using a human fetal brain cDNA library. From this library, three independent positive clones, pACT2-H-1, -2, and -3, were isolated which encoded the C-terminal portions of MAP4K4 (Fig. 1A). MAP4K4 contains the N-terminal kinase domain bearing homology to STE20, the intervening region that includes a coiled-coil domain, and the C-terminal regulatory domain termed the CNH domain. The CNH domain was encoded by all the three clones, suggesting that it interacts with Rap2. Several Ras-interacting proteins contain regions of about 100 amino acids that mediate interactions with Ras; the Ras-binding domain and the Ras-associating domain (1). However, these domains were not found within the CNH domain of MAP4K4. When the CNH domain was divided into N- and C-terminal portions, each portion did not interact with Rap2 (Fig. 1A). This suggests that the entire CNH domain is required for interaction with Rap2. Alternatively, a region mediating the interaction may overlap the boundary between the two portions.

Next, we examined the interaction of MAP4K4 with Rap2 mutants and other small GTP-binding proteins by the two-hybrid assay (Table I). MAP4K4 strongly interacted with Rap2 and its activated mutant, Rap2(G12V), while it interacted weakly with the dominant negative mutant, Rap2(S17N). MAP4K4 barely interacted with the effector region mutant, Rap2(F39S). MAP4K4 failed to interact with Rap1, its activated mutant, Rap1(G12V), or Ras. In comparison, Raf-1 failed to interact with only the dominant negative mutant, Rap2(S17N). Both MAP4K4 and Raf-1 failed to in-
MAP4K4 as an Effector of Rap2

Specific interaction of MAP4K4 with Rap2 in the yeast two-hybrid assay

The C-terminal portion of MAP4K4 (amino acids 711–1212 encoded by pACT2-H-1) and the N-terminal portion of Rap1 (amino acids 48–300 encoded by pGAD-Raf-1) (10) were examined for their interaction with wild-type Rap2A, its activated (G12V), dominant negative (S17N), and effector region (F39S) mutants, wild-type Rap1A, its activated (G12V) mutant, and wild-type Ha-Ras encoded by respective pBTM116 constructs in the L40 strain.

| pACT2-H-1 | pGAD-Raf-1 | pACT2 |
|-----------|------------|-------|
| HIS3a      | β-Galb     | HIS3 | β-Gal |
| pBTM116-Rap2A | + + | 53.7 | + + | 78.9 | 0.8 |
| pBTM116-Rap2A(G12V) | + + | 54.9 | + + | 83.4 | 0.9 |
| pBTM116-Rap2A(S17N) | ± | 18.5 | ± | 4.3 | 2.3 |
| pBTM116-Rap2A(F39S) | ± | 7.1 | ± | 95.6 | 1.7 |
| pBTM116-Rap1A | – | 1.6 | – | 53.3 | 1.2 |
| pBTM116-Rap1A(G12V) | – | 3.6 | + + | 55.7 | 2.1 |
| pBTM116-Ha-Ras | – | 2.8 | + + | 144.8 | 2.7 |

a Co-transformants were examined for HIS3 expression: ++, strong expression; ±, weak expression; –, no expression.
b Co-transformants were also examined for β-galactosidase (β-Gal) expression by the o-nitrophenyl-β-D-galactopyranosidase assay (7).

MAP4K4 activates JNK (2, 3). We therefore tested whether Rap2 affects this process. FLAG-tagged JNK (FLAG-JNK2) was expressed alone, with HA-MAP4K4, with Myc-Rap2A, or with both of them in 293T cells (Fig. 3). Activation of JNK was examined by assessing the extent of phosphorylation of JNK within cell homogenates by Western immunoblotting with anti-
MAP4K4 as an Effector of Rap2

DISCUSSION

The functional difference between Rap2 and its close relative Rap1, sharing 60% amino acid identity, has been unclear. In other words, it has not been clear why Rap2 in addition to Rap1 exists. One possible answer is that Rap1 and Rap2 interact with different effectors and regulate different signaling pathways. Consistent with this, we have identified in the present study MAP4K4 as a putative effector of Rap2 that does not interact with Rap1. The effector region of Rap2 is different from that of Rap1 by a single amino acid, Phe-39. The importance of this amino acid in specific interaction with MAP4K4 was demonstrated by the substitution of Phe with Ser, present in the corresponding position in Rap1, which resulted in severe attenuation of interaction. The interaction may be conserved among mult cellular organisms, as the C. elegans ortholog of MAP4K4 (MIG-15) interacts with the C. elegans ortholog of Rap2 (C25D7.7 protein) but not with orthologs of Rap1 (C27B7.8 protein) or Ras (LET-60)2.

Rap2 interacts with the CNH domain of MAP4K4. The CNH domain was originally found in citron that interacts with Rho and Rac (11). Subsequently, it was found in Rom2p, a GDP/GTP exchange factor for S. cerevisiae Rho protein (Rho1p), and the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCKα) (12, 13). Although all three proteins interact with Rho family small GTP-binding proteins, their CNH domains do not mediate the interactions directly. The CNH domain is also present in the C-terminal portions of GCK-I and -IV subfamilies of the STE20 group kinases (5, 6). GCK/MAP4K2, which belongs to the GCK-I subfamily, interacts with Rab8 small GTP-binding protein through its C-terminal portion containing the CNH domain (14). GCK/MAP4K2 interacts with the GTP-bound form of Rab8 preferentially over the GDP-bound form, and the interaction requires the intact effector region of Rab8. This suggests that GCK/MAP4K2 might act as an effector of Rab8 (14). Since our present study indicated that the CNH domain in MAP4K4 interacts with Rap2, it is interesting to hypothesize that some of the CNH domains in the STE20 group kinases may share a region that mediates interactions with small GTP-binding proteins.

Acknowledgments—We thank Dr. Katsuyoshi Yoshioka and Dr. Takahiro Nagase for plasmids. We also thank Kyoko Kise for help in preparation of the manuscript, Asako Umikawa for technical assistance, and Makiko Gushi for discussion.

REFERENCES

1. Campbell, S. L., Khorasavi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1413
2. Yao, Z., Zhou, G., Wang, X. S., Brown, A., Diener, K., Gan, H., and Tan, T.-H. (1999) J. Biol. Chem. 274, 21186–2125
3. Su, Y.-C., Han, J., Xu, S., Cobb, M., and Skolnik, E. Y. (1997) EMBO J. 16, 1279–1290
4. Wright, J. H., Wang, X., Manning, G., LaMere, B. J., Le, P., Zhu, S., and Khatry, D., Flanagan, P. M., Buckley, S. D., Whyte, D. B., Howlett, A. R., Bischoff, J. R., Lipson, E. K., and Jaffal, B. (2003) Mol. Cell. Biol. 23, 2068–2082
5. Kyrkaia, J. M. (1999) J. Biol. Chem. 274, 5259–5262
6. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) Trends Cell Biol. 11, 220–230
7. Kisho, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Furuta, H., Hotta, K., Kodita, K., Watanabe, T., Ohya, Y., and Takai, Y. (1996) EMBO J. 15, 6066–6076
8. Umikawa, M., Obashi, H., Nakanishi, H., Sato-Horikawa, K., Takahashi, K., Hotta, I., Matsumura, Y., and Takai, Y. (1999) J. Biol. Chem. 274, 25197–25200
9. Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppa, Y., Shiba, T., and Yamamoto, K. I. (1999) Mol. Cell. Biol. 19, 7529–7548
10. Sendoh, H., Hu, C.-D., Wu, D., Song, C., Yamawaki-Katoaka, Y., Kotsuji, N., Okada, T., Shima, F., Kariya, K., and Katoaka, T. (2000) Biochem. Biophys. Res. Commun. 271, 596–602
11. Madjdane, P., Furuyaishi, T., Reid, T., Ichizaki, T., Watanabe, G., Morii, N., and Narumiya, S. (1995) FEBS Lett. 377, 243–248
12. Ozaki, K., Tanaka, K., Imamura, H., Hihata, T., Kameyama, T., Nonaka, H., Hirano, H., Matsumura, Y., and Takai, Y. (1996) EMBO J. 15, 2201–2207
13. Chen, X.-Q., Tan, I., Leung, T., and Lim, L. (1999) J. Biol. Chem. 274, 19901–19905
14. Ren, M., Zeng, J., De Lemus-Chiardradini, C., Rosenfeld, M., Aidesnik, M., and Sabattini, D. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5151–5155

2 M. Oshiro and K. Kariya, unpublished observation.
