DRAKs, Novel Serine/Threonine Kinases Related to Death-associated Protein Kinase That Trigger Apoptosis*

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The present study describes the cloning of two novel serine/threonine kinases termed DRAK1 and DRAK2, whose catalytic domains are related to that of death-associated protein kinase, a serine/threonine kinase involved in apoptosis. Both DRAKs are composed of the N-terminal catalytic domain and the C-terminal domain that is responsible for regulation of kinase activity. DRAK1 and DRAK2 show 59.7% identity and display ubiquitously expressed. An in vitro kinase assay revealed that both DRAKs are autophosphorylated and phosphorylated myosin light chain as an exogenous substrate, although the kinase activity of DRAK2 is significantly lower than that of DRAK1. Both DRAKs are exclusively localized to the nucleus. Furthermore, overexpression of both DRAKs induces the morphological changes of apoptosis in NIH 3T3 cells, suggesting the role of DRAKs in apoptotic signaling.

Apoptosis is a highly regulated active process of cell death that plays a crucial role in development and tissue homeostasis, but its deregulation is associated with the pathogenesis of a number of diseases including autoimmune disease, neurodegenerative disorders, and cancers (1–3). Cells undergoing apoptosis show characteristic morphological changes, including membrane blebbing, chromatin condensation, and DNA fragmentation. Although the mechanisms that control the process of apoptosis are still poorly understood, an increasing number of regulatory molecules involved in apoptosis have been currently identified and characterized. They include caspase family of cysteine protease, the Bcl-2 family members, as well as tumor suppressor and oncogenic proteins such as p53, c-Myc, c-Jun, and RB (4–12). The evidence that protein kinases may participate in regulation of apoptosis is also now accumulating (13). One serine/threonine kinase, termed ZIP kinase, was recently cloned by our laboratory through the yeast two-hybrid system in which the leucine zipper domain of ATF4, a member of activating transcription factor/cAMP-responsive element binding protein (ATF/CREB) family of transcriptional factors, was used as a bait (14). In addition to an N-terminal serine/threonine kinase domain, ZIP kinase contains a C-terminal leucine zipper domain which is sufficient not only for the homodimerization and kinase activation but also for binding to ATF4. Overexpression of wild type ZIP kinase, but not kinase inactive mutant, caused the morphological changes typical of apoptosis in NIH 3T3 cells, suggesting that the kinase activity of ZIP kinase triggers apoptosis. Furthermore, the catalytic domain of ZIP kinase shares homology to that of DAP\(^3\) kinase. DAP kinase is also found to participate in apoptosis since its reduced expression by antisense mRNA protected HeLa cells from apoptosis induced by interferon-\(\gamma\) (15). In addition, overexpression of DAP kinase induced apoptosis, and kinase-negative mutant blocked apoptosis by interferon-\(\gamma\) (16).

This study describes the cloning and characterization of two additional members of DAP kinase family, which we termed DRAK1 and DRAK2. Overexpression of DRAK1 and DRAK2 induced the morphological changes of apoptosis, as did DAP kinase and ZIP kinase. Therefore, we propose that these kinases form a new family of protein kinases that mediate apoptosis.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Northern Blot Analysis—A partial cDNA of the EST clone encoding DRAK1 was obtained by reverse transcriptase-PCR from a human placenta cDNA. To obtain full-length of DRAK1 cDNA, a human placenta Agt11 cDNA library (CLONTECH) was screened by hybridization through the standard procedures. DNA inserts of the purified phage clones were characterized by restriction enzyme mapping and nucleotide sequencing using an automated DNA sequencer (ABI model 377). Full-length of the DRAK2 cDNA was obtained by the rapid amplification of cDNA ends protocol from a human liver cDNA library according to the manufacturer’s instructions (CLONTECH).

Full-length cDNAs of human DRAK1 and DRAK2 were used to probe human Multiple Tissue Northern blots containing 2 \(\mu\)g of polyA\(^1\) RNA isolated from various tissues according to the manufacturer’s instructions (CLONTECH).

Plasmids—N-terminal FLAG- or Myc-tagged DRAK cDNAs were obtained by PCR. The PCR products were subcloned into pEF-BOS, a mammalian expression plasmid (17). The site-directed mutagenesis was performed using Transformer Site-directed Mutagenesis Kit (CLONTECH). Sequences of all constructs were confirmed by DNA sequencing. Sequences of the primer are available upon request.

In Vitro Kinase Assay—One million of COS-7 cells were transiently transfected with 10 \(\mu\)g of the plasmid by Lipofectin (Mirus Corp.). Cells were lysed with lysis buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. Cell lysates were immunoprecipitated with protein-G Sepharose (Amersham Pharmacia Biotech) together with 10 \(\mu\)g/ml anti-FLAG M2 antibody (Eastman Kodak Co.). Immunoprecipitates were washed four times with lysis buffer and once with kinase reaction buffer (10 mM MgCl\(_2\), 3 mM MnCl\(_2\), 10 mM Tris-HCl, pH 7.2). In the in vitro kinase reaction was carried out in kinase reaction buffer containing 10 \(\mu\)Ci of [\(^{32}\)P]ATP (Amersham Pharmacia Biotech) for 10 min at 30 °C in the presence of 5 \(\mu\)g of myosin light chain (Sigma). Laemmli sample buffer was added to terminate the kinase reaction. After boiling, the samples were separated on SDS-polycrylamide gel electrophoresis and then visualized by autoradiography.

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¶ The abbreviations used are: DAP, death-associated protein; PCR, polymerase chain reaction; mAb, monoclonal antibody; MLIC, myosin light chain; X-gal, 5-bromo-4-chloro-3-indolyl \(\beta\)-galactopyranoside; WT, wild type; TNF, tumor necrosis factor; DAPI, 4,6-diamidino-2-phenylindole; CaMK, calcium/calmodulin-dependent protein kinase.
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RESULTS

Cloning and Tissue Distribution of Human DRAK1 and DRAK2—To isolate genes that harbor the region homologous to the catalytic domains of DAP and ZIP kinases, we searched for DAP kinase-related EST clones using the BLAST and FASTA programs. The searches identified two novel genes that show a high degree homology with DAP kinase. Full-length cDNAs were isolated from human placenta and liver cDNA libraries by a combination of hybridization and PCR techniques. We termed these molecules DAP kinase-related apoptosis-inducing protein kinase (DRAK)1 and DRAK2. Nucleotide sequence analyses revealed that DRAK1 contains an open reading frame of 414 amino acids with a predicted molecular mass of 46.56 kDa, whereas DRAK2 contains an open reading frame that encodes 372 amino acids with a mass of 42.34 kDa (Fig. 1). The putative kinase domains of both DRAKs are located at the N terminus and contain all 11 subdomains conserved among all serine/threonine kinases (Figs. 1 and 2) (18). Within the kinase domain, DRAK1 shows the highest homology to DRAK2 (67.1% identity). Besides the kinase domain, there is a homology in the non-catalytic C-terminal regions between DRAK1 and DRAK2 with 24.2% identity. These domains, however, do not reveal any significant homology either to those of DAP and ZIP kinases or to any other proteins. Within the kinase domain, DRAKs also display homology to CaMK II, and MLC kinase, in the catalytic domains of DAP and ZIP kinases. DAP kinase is tightly associated with the cytoskeleton, whereas ZIP kinase is localized to the nucleus. Therefore, we investigated the cellular localizations of both DRAKs. The expression plasmid for either FLAG-tagged DRAKs WT or the kinase negative mutants (DRAK1 K90A, DRAK2 K62A) was transiently transfected into COS-7 cells and subjected to the in vitro kinase assay. As shown in Fig. 4C, both auto- and MLC phosphorylations were detected in cells transfected with the deletion mutant encoding DRAK1 amino acids 1–345 (Myc-DRAK1-(1–345)), with the same level of wild type DRAK1. By contrast, the kinase activity was drastically decreased in cells expressing the mutant encoding only the kinase domain of DRAK1 (Myc-DRAK1-(1–321)), suggesting the critical role of the C terminus for full activation of the kinase. Unlike DRAK1, the kinase activity of the mutant DRAK2 that lacked the C terminus (Myc-DRAK2-(1–293)) was higher than in cells introduced full-length DRAK2 or C-terminal deleted mutant (amino acids 1–315) of DRAK2. However, we found that the kinase activity of the mutants encoding only the kinase domain is roughly equal between DRAK1 and DRAK2 (data not shown). These results suggest the opposing function of the C terminus of both DRAKs in modulation of the kinase activity despite their similar structures.

Cellular Localization of DRAK1 and DRAK2—It was demonstrated that the cellular localization is different between DAP and ZIP kinases. DAP kinase is tightly associated with the cytoskeleton, whereas ZIP kinase is localized to the nucleus. Therefore, we investigated the cellular localizations of both DRAKs. The expression plasmid for either FLAG-tagged DRAKs WT or the kinase negative mutants (DRAK1 K90A, DRAK2 K62A) was transiently transfected into COS-7 cells. After 36 h, the FLAG proteins were detected by indirect immunostaining with anti-FLAG mAb and fluorescein-conjugated secondary antibody. The nuclei were simultaneously visualized by staining with DAPI. As shown in Fig. 5, the fluorescent signals for DRAKs and the nucleus overlapped, indicating that both DRAKs are exclusively localized to the nucleus, and the kinase activity was not required for the nuclear localization. However, we found that DRAKs were localized to both cytoplasm and nucleus in some transfected cells.
Apoptosis Induced by DRAKs—DAP kinase is involved in the apoptotic process initiated by the interferon-γ receptor signaling (15, 16). ZIP kinase also induces apoptosis of NIH 3T3 cells when it is overexpressed (14). Therefore, we tested the ability of DRAKs to induce apoptosis. NIH 3T3 mouse fibroblast cells were transiently co-transfected with the expression plasmid for either DRAK1 or DRAK2 together with the LacZ expression plasmid as an indicator. Transfected cells were visualized by staining with X-gal solution. As seen in Fig. 6, blue-stained cells transfected with wild type DRAK1 or DRAK2 display the morphological changes of typical apoptosis characterized by shrunken cell size and membrane blebbing (DRAK1, 25.6%; DRAK2, 23.4%). In contrast, these features are not observed in the transfectants with catalytically negative mutants of DRAKs (DRAK1 K90A, 1.1%; DRAK2 K62A, 2.0%). In addition, the phenotypes of these mutant transfectants were almost the same with mock transfectants used as a control (data not shown).

To investigate that there is some correlation between the kinase activity and initiation of apoptosis, we carried out the colony formation assay with the expression plasmid for DRAKs or their mutants. Each construct (Myc-DRAK1 WT, Myc-DRAK1-(1–321), FLAG-DRAK1 K90A, Myc-DRAK2 WT, Myc-DRAK2-(1–293), FLAG-DRAK2 K62A, or empty vector) was co-transfected along with neomycin resistance gene (pSV2 neo) into NIH 3T3 cells. Three weeks after transfection, G418-resistant colonies were scored by staining with 0.1% crystal violet solution. As seen in Table I, the colony numbers of both DRAKs WT were significantly reduced when compared with the vector transfected. In contrast, the colony numbers of the kinase negative mutants of both DRAKs were almost same with the vector transfected. In the case of the deletion mutants encoding only the kinase domains of both DRAKs (DRAK1-(1–321) and DRAK2-(1–293)), the number of colonies was increased when compared with the wild type of DRAKs. These results suggest that cell death-inducing activity of DRAKs was dependent on their intact structures but not their intensities of the kinase activity, and the C-terminal domain of DRAKs was necessary for induction of apoptosis. Furthermore, the cell death-inducing activity was similar for both DRAKs because of almost the same colony number between DRAK1 WT and DRAK2 WT transfections.

DISCUSSION

Protein kinases play critical roles in the signal transduction in response to a number of external stimuli. However, the role for kinases in apoptosis is not clear. This study describes the
cDNA cloning and characterization of two novel serine/threonine kinases designated DRAK1 and DRAK2. Both DRAKs are closely related throughout their overall structures. Furthermore, ectopic expression of DRAKs, but not kinase inactive DRAKs, triggered morphological changes typical of apoptosis of NIH 3T3 cells, suggesting the catalytic activities of both DRAKs to link the apoptotic process. The kinase domains of both DRAKs share homology to that of DAP kinase, which is involved in the apoptotic signaling induced by interferon-γ.

Reduced expression of DAP kinase by antisense mRNA inhibits apoptosis by interferon-γ (15). Overexpression of DAP kinase induced apoptosis, and the catalytically inactive mutant blocked apoptosis by interferon-γ (16). Recently, we have identified ZIP kinase whose kinase domain is the most closely related to that of DAP kinase (14). Overexpression of ZIP kinase also induced apoptosis of NIH 3T3 cells as did DRAKs. Taken together, these kinases may form a new family of serine/threonine kinase that mediates apoptosis.

Although kinase domains among DRAKs, DAP kinase, and ZIP kinase are closely related, the non-catalytic C-terminal regions are structurally different and do not share any homology. DAP kinase contains two known domains characterized by eight ankyrin repeats and the death domain, whereas ZIP kinase has the leucine zipper domain at its C-terminal end. These domains are known to mediate protein-protein interactions. In fact, ZIP kinase is activated when it homodimerizes through the leucine zipper domain. In case of DAP kinase, apoptosis was not induced in cells ectopically expressing the mutant that lacks the C-terminal region containing the ankyrin repeats and the death domain. This suggests that DAP kinase may be activated by formation of homodimer or binding to the regulatory molecule(s) through interactions with the domains. Similarly, the non-catalytic C-terminal regions of both DRAKs may also function as an interaction domain, although there is no significant homology to any other protein.

The transfection studies with the DRAKs expression vectors indicated that the C-terminal domains of both DRAKs may function as an interacting domain with a regulator or a specific substrate for mediating apoptosis. cDNA cloning and characterization of two novel serine/threonine kinases designated DRAK1 and DRAK2. Both DRAKs are closely related throughout their overall structures. Furthermore, ectopic expression of DRAKs, but not kinase inactive DRAKs, triggered morphological changes typical of apoptosis of NIH 3T3 cells, suggesting the catalytic activities of both DRAKs to link the apoptotic process. The kinase domains of both DRAKs share homology to that of DAP kinase, which is involved in the apoptotic signaling induced by interferon-γ.

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diating apoptosis. Surprisingly, the induction of cell death was higher in cells expressing the wild type DRAK2 than DRAK2-(1–293), although the kinase activity of wild type DRAK2 was lower than DRAK2-(1–293). Therefore, there was no correlation between the intensity of the kinase activity and the induction of apoptosis. However, the kinase activity in addition to C-terminal domain was also required for the induction of apoptosis because the kinase-inactive DRAKs failed to induce apoptosis.

We found that overexpression of both DRAKs did not cause the morphological changes of apoptosis in COS-7 cells (data not shown), which is different from the NIH 3T3 cells. In fact, the nucleus exhibited normal morphology when DRAKs were overexpressed into COS-7 cells (Fig. 5). It was considered that the genetic changes that protect cells from DRAKs-induced apoptosis occurred in the immortalization process of COS-7 cells or that the sensitivity to DRAKs-induced apoptosis was different depending on the cell type.

Based on the sequence analysis, we found that there is a stretch of basic amino acids in the kinase subdomain II in DRAKs, ZIP kinase, and DAP kinase (Fig. 2). It is possible that the nuclear localization of DRAKs depends on the sequences, because the deletion mutant lacking the non-catalytic C-terminal region of ZIP kinase was exclusively localized to the nucleus (14). In addition, the mutant DAP kinase lacking the C terminus composed of ankyrin repeats and the death domain was localized to nucleus, although the intact form was tightly associated with the cytoskeleton (16). It is possible that these kinases in some way function in the nucleus where they phosphorylate the downstream targets of the apoptotic machinery. However, upstream regulators or specific substrates for DAP kinase and ZIP kinase have not been identified.

It is important to understand the regulation and function of DRAKs on apoptosis. Previously, several kinases besides DAP and ZIP kinases are reported to be activated in response to various apoptotic stimuli such as TNF-α, FasL, UV light, and certain drugs. JNKs, members of mitogen-activated protein kinase family, are reported to be activated by various apoptotic stimuli, whereas the blockage of JNKs pathway inhibited cells from apoptosis by such stimuli (19, 20). Furthermore, upstream kinases of JNKs, ASK1, and MEKK1 were also known to induce apoptosis upon overexpression (21–23). Recently, it was reported that activation of CaMK II is an essential event in TNF-α- and UV light-induced apoptosis (24). CaMK II was rapidly activated in Ca^{2+}-independent manner in response to TNF-α or UV light, and apoptosis was suppressed by CaMK II inhibitors. Considering that DRAKs are structurally related to CaMK II, DRAKs may be activated in response to various apoptotic stimuli as described above.

Recent study has implicated that DAP kinase functions as a tumor suppressor gene (25, 26). The lung carcinoma clones, which behave as high metastatic cells, did not express DAP kinase, in contrast to cells behaving as low metastatic cells.
When the expression of DAP kinase was restored into high metastatic carcinoma cells, the ability of lung metastasis was significantly suppressed after injection of cells into mice. Furthermore, the susceptibility to apoptosis in response to TNF was increased in the DAP kinase transfectants in vitro. We are currently investigating the expression of DRAK1 and DRAK2 in various tumor cells and their roles in tumorigenesis and metastasis.

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