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Biochemistry

Newly identified stress-responsive protein kinases, Krs-1 and Krs-2

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ABSTRACT **The activation of protein kinases is a frequent response of cells to treatment with growth factors, chemicals, heat shock, or apoptosis-inducing agents. However, when several agents result in the activation of the same enzymes, it is unclear how specific biological responses are generated. We describe here two protein kinases that are activated by a subset of stress conditions or apoptotic agents but are not activated by commonly used mitogenic stimuli. Purification and cloning demonstrate that these protein kinases are members of a subfamily of kinases related to Ste20p, a serine/threonine kinase that functions early in a pheromone responsive signal transduction cascade in yeast. The specificity of Krs-1 and Krs-2 activation and their similarity to Ste20p suggest that they may function at an early step in phosphorylation events that are specific responses to some forms of chemical stress or extreme heat shock.**

It is critical for all organisms to sense extracellular signals and generate an appropriate biological response. In eukaryotes, extracellular cues often drive the activation of protein kinases, and acute changes in kinase activity are responsible for initiating the resulting phenotypic alterations (1, 2). Although the stimulation of protein kinase cascades by mitogenic stimuli and oncogenes has been long recognized, it is now clear that cells respond to stress such as heat shock or chemical treatment by activating protein kinases and expressing heat shock proteins (3, 4). These alterations presumably allow cells to resist the unfavorable environmental conditions, and such physiological changes likely occur in response to injury and disease (5).

Map kinase was initially identified in mammalian cells as an enzyme that requires tyrosine and threonine phosphorylation for activation (6). Map kinase in mammalian cells functions downstream of the kinases Raf and Mek, respectively, in a pathway that receives signals from effectors acting through the plasma membrane (reviewed in refs. 1, 4, and 7). Protein kinase pathways using enzymes related to Map kinase, Mek, and either Raf or Mekk, another class of Mek activators, are found in mammals, flies, yeast, and plants (reviewed in ref. 8). In mammalian cells, the pathway initially characterized is activated primarily by growth-promoting stimuli. Members of the Map kinase family have more recently been shown to be activated under a variety of stress conditions, however (reviewed in ref. 1 and 4). In *Saccharomyces cerevisiae,* pathways using enzymes with sequence similarities to mammalian participants in the Map kinase pathway are involved in pheromone response, cell wall construction, osmosensing, and spore formation (reviewed in refs. 4 and 7). The protein kinase encoded by *STE20* acts upstream of the Mekk position in the pheromone-responsive Map kinase pathway and perhaps participates in other pathways (9–11).

In mammalian cells, Jnk (also called Sapk), a Map kinase family member, is activated by environmental stress and apoptosis-inducing agents (12–14). Another Map kinase-related enzyme, p38, is also activated by environmental stress; however, this enzyme appears to lie in a distinct protein kinase pathway (15–17). p38 is activated by distinct Mek homologs Mkk3 and Mkk6, whereas Jnk is activated by the Mek homolog Mkk4 (18–20). This specificity may extend to the Ste20p homolog, germinal center kinase, which has been reported to activate Mkk4 and Jnk but not p38 (21). It is unclear, however, how these specific phosphorylation responses are controlled, as both Jnk and p38 are activated in cells by identical stress stimuli.

In addition to their roles as upstream activators of Map kinase pathways, members of the Ste20p family may also function in the control of cellular morphology. Bem1p, a protein in *S. cerevisiae* required for establishment of cell polarity during mating, interacts with Ste20p and actin (11, 22). Moreover, some Bem1p mutants that result in defective polarity establishment maintain their interaction with actin but fail to associate with Ste20p, suggesting that Ste20p may play an essential role in polarity regulation. Data suggest that Sps1p and Cla4p, other Ste20p family members in *S. cerevisiae*, also function in the control of morphology (23, 24). In *Schizosaccharomyces pombe*, overexpression of an inactive form of the protein product of the *PAK1* gene, another Ste20p family member, results in abnormally shaped cells and disruption of actin localization (25). A recently identified *Drosophila* homolog of Ste20p, DPak, colocalizes with focal adhesions and focal complexes in the developing embryo (26). In addition, the putative upstream activators of some mammalian Ste20p homologs, members of the Rho subfamily of GTPases, play specialized roles in regulating the cytoskeletal alterations in response to extracellular cues (27–29).

We have previously identified a 63-kDa protein kinase activity that is activated late in the transformation of chicken embryo fibroblasts by $pp60^{v\text{-}src}$, and its activity correlated with the presentation of the transformed cell phenotype (30). This activity was also observed in cells treated with okadaic acid (OA). Subsequently, a similar activity was detected in mammalian cells after treatment with staurosporine (STR) or OA (H,-C.R.W., L.K.T., G. Rajgolikar, and R.L.E., unpublished data). In this communication, we report the purification of the enzyme responsible for this activity from mammalian cells. Peptide sequence data from this enzyme and a copurifying 61-kDa protein kinase were used to obtain cDNAs corresponding to the mRNAs for the enzymes. These protein kinases proved to be related to yeast Ste20p and Sps1p, as well as to several recently cloned mammalian enzymes (9, 10, 23, 31–34). The homology to Ste20p and the specificity of activation of these enzymes, which we propose to be denoted Krs-1 (63 kDa)

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Abbreviations: OA, okadaic acid; STR, staurosporine; HA, hemagglutinin; MBP, myelin basic protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U60206 and U60207).

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and Krs-2 (61 kDa) for *k*inases *r*esponsive to *s*tress, suggest they may function early in a mammalian cell stress response pathway.

MATERIALS AND METHODS

Materials. The αKrs antibody was generated against aa 301–322 in Krs-2 by the PolyQuik method (Zymed). The αhemagglutinin (HA) antibody was prepared from mouse ascites fluid, and the horseradish peroxidase-conjugated antibodies were obtained from Amersham. STR was obtained from Kamiya Biomedical (Thousand Oaks, CA). [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN. Chromatography resins and columns were purchased from Pharmacia, with the exception of the phenyl Sepharose resin, which was obtained from Sigma.

Treatment of NIH 3T3 Cells. NIH 3T3 cells were cultured in DMEM supplemented with 5% calf serum and 100 units/ml penicillin/streptomycin on 100-mm tissue culture plates. The cells at 80% confluency remained untreated or were treated in culture medium with either 0.5 mM or 250 mM sodium *m*-arsenite, 10 μM carbonyl cyanide *m*-chlorophenylhydrazone, 400 mM NaCl or 600 mM sorbitol at 37°C for 15 min, 1 μM OA or 1 μM STR at 37°C for 1 hr, heat shocked at 45°C or 55°C for 15 min or UV-irradiated for 1 min, followed by incubation at 37°C for 1 hr. The cells were then washed once with PBS, scraped from the plate in 1 ml PBS, and pelleted in a microfuge for 30 sec. The cell pellet was resuspended in 150 μl of TEV+ [20 mM Tris, pH 7.4/1 mM EGTA, pH 7.4/100 μM sodium orthovanadate/10 μg/ml leupeptin and pepstatin/0.02 unit/ml aprotinin/1 mM Pefabloc SC (Boehringer Mannheim)/10 mM *p*-nitrophenol phosphate], and the cells were lysed by sonication. Insoluble material was pelleted with a 30 min centrifugation at 12,000 × *g* at 4°C, and the resulting supernatant was collected. The protein concentration in each sample was determined by the Bradford method (35), and the remaining supernatant was combined with Laemmli sample buffer (36) and boiled in preparation for the in-gel kinase assay.

In-Gel Kinase Assay. Either equal amounts of protein or equal volumes from immunoprecipitations or column fractions were applied to an SDS/10% polyacrylamide gel copolymerized with 0.4 mg/ml myelin basic protein (MBP). The gel was processed for the in-gel kinase assay as previously described with 50 μM unlabeled ATP and 25 μCi/ml [γ-^{32}P]ATP (30, 37). After drying, the gel was subjected to autoradiography on Biomax film (Kodak).

Purification of p61 and p63. T-cell hybridomas were grown in DMEM supplemented with 5% calf serum/5% fetal calf serum/0.03% glutamine/10 mM Hepes, pH 7.1/100 units/ml penicillin/streptomycin to 1–2 × 10^6 cells/ml. Cells from 6 liters of culture were collected, pelleted, resuspended in 160 ml of culture medium, and treated for 1 hr with 1 μm STR. The cell pellets were quick-frozen in liquid nitrogen and stored at −70°C. The cell pellets from 8 liters of hybridoma culture were resuspended in 125 ml of TEV+ with 10 nM OA, and the cells were homogenized with a Tissumizer (Tekmar, Cincinnati) followed by 25 strokes with a tight fighting pestle in a Dounce homogenizer. After centrifugation for 30 min at 100,000 × *g* at 4°C, the supernatant was loaded onto a 20-ml Fast Flow Q column preequilibrated in TEV (20 mM Tris, pH 7.4/1 mM EGTA, pH 7.4/100 μm sodium orthovanadate). The migration of p63 activity was monitored in all chromatography steps by the MBP in-gel kinase assay. The p63 activity was eluted from the column in a 0.2–0.4 M NaCl step, dialyzed against TEV, and loaded onto a 6-ml Resource Q column. The column was developed with a 75-ml, 0–0.5 mM NaCl gradient at a flow rate of 4 ml/min, and p63 activity eluting at 0.3 M NaCl was pooled and loaded onto a 1-ml phenyl Sepharose column previously equilibrated with 0.3 M NaCl in TEV. The column was run at 1 ml/min, and p63 activity eluted at the tail of a gradient of 0–60% ethylene glycol in TEV. This purification paradigm was repeated three times.

The peaks of p63 activity from the previous purifications were pooled and loaded onto a Mono Q anion exchange column on a fast protein liquid chromatography (Pharmacia) column previously equilibrated in TEV. The column was developed with a 20 ml 0.25–0.4 M NaCl gradient at a flow rate of 1 ml/min. The p61/p63 activity was detected by the in-gel kinase assay.

Peptide Microsequencing. Following the final Mono Q column, fractions enriched in p61 (fractions 45–48) and p63 (fractions 50–53) were pooled and concentrated in Microcons (Amicon). Approximately 50% of the purified protein from each pool was resolved on a 10% polyacrylamide gel, transferred to poly(vinylidene difluoride) membrane (Bio-Rad), and digested *in situ* with trypsin (38). The tryptic peptides were separated by HPLC and sequenced using an automated peptide sequencer as described (39).

Isolation of cDNAs. One degenerate oligonucleotide primer was synthesized based upon the amino acid sequence obtained from sequencing of peptide 1 in p61, GGAATTCCGG(C/A/T/G)(A/T)(C/G)(C/A/T/G)TA(C/T)GG(C/A/T/G)(A/T)(C/G)(C/A/T/G)GT(C/A/T/G)TA(C/T)AA. A second primer was made complementary to the sequence of a PCR product designated HsPK23 previously deposited in the GenBank data base (40), CGGGATCCCG^{303}GGTACCCTGAATCACTTCTGG^{283}. These oligonucleotides (1 μM) were used in a PCR containing 0.5 mM deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, 1× Promega *Taq* buffer, 1 unit of *Taq* polymerase, and a sample of human cDNAs (provided by M. Baron, Harvard University, Cambridge, MA). Thirty cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 1 min were preceded by a 10-min incubation at 94°C and followed by a 10-min extension at 72°C. The resulting heterogeneous 400- to 600-bp product was reamplified under the same conditions, and this amplification yielded a single band of ≈500 bp. This product was cloned into Bluescript KS$^-$ and sequenced to determine that it contained the predicted product. The ^{32}P-labeled 486-bp insert was used to probe a human cDNA library made from Jurkat cells (obtained from S. Schreiber, Harvard University). Hybridization was performed at 37°C for 72 hr in 0.5 M sodium phosphate, pH 7.2/1 mM EDTA/1% BSA/7% SDS. The filters were washed for 1 hr at 37°C in 20 mM sodium phosphate, pH 7.2/1 mM EDTA/1% SDS, and labeled plaques were detected by autoradiography. The complete coding sequence for Krs-1 was contained within a single clone, H33. The complete coding sequence for Krs-2 was derived from two clones, H22 and H243.

Immunoprecipitation. T-cell hybridomas (2 × 10^6 cells/ml, 100 ml) remained untreated or were treated with 1 μm STR for 1 hr. The cells were washed two times with PBS, and the cell pellet was resuspended in 1 ml of TEV+ and sonicated to lyse the cells. Insoluble material was pelleted by a 30-min centrifugation at 12,000 × *g* at 4°C, and protein concentrations were determined by the Bradford method. The cell lysates were then either combined with Laemmli sample buffer and boiled in preparation for direct Western analysis or in-gel kinase assays, or 4.6 mg of cell lysate was combined with 1 ml of RIPA+ buffer (10 mM Tris, pH 8.0/1 mM $MgCl_2$/50 mM NaCl/10% glycerol/1% Nonidet P-40/100 μM sodium orthovanadate/10 μg/ml leupeptin and pepstatin/0.02 unit/ml aprotinin/1 mM Pefabloc SC/10 mM *p*-nitrophenol phosphate) and 2 μg of the αKrs antibody that had been previously incubated on ice for 30 min in the presence or absence of 2 μg of the immunizing peptide. Incubation with the primary antibody was conducted for 2 hr at 4°C, followed by incubation with 25 μl of protein A agarose (Zymed) for 30 min at 4°C. The protein A pellets were washed once with RIPA+ and two times with TEV, and then

resuspended in TEV+. The samples were combined with Laemmli sample buffer and boiled before Western analysis and in-gel assays.

For Western blot analysis, 40 μg of cell lysate or 33% of each immunoprecipitate was resolved on a SDS/9% polyacrylamide gel and transferred to Immobilon-P (Millipore). The blot was blocked in TBS-T (20 mM Tris, pH 7.6/137 mM NaCl/0.1% Tween-20/6% BSA) and probed with 0.25 μg/ml αKrs antibody in TBS-T followed by an horseradish peroxidase-conjugated donkey α-rabbit antibody. Immunoreactive species were detected by enhanced chemiluminescence (Amersham). The same quantities of cell lysate and immunoprecipitations were used for the in-gel kinase assay.

Transient Transfections. The cDNAs encoding Krs-1 and Krs-2 were tagged at the C terminus with the HA tag and cloned into the pC1neo vector (Promega). The human kidney cell line 293 was grown in DMEM supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin. Approximately 30 hr before transfection 4×10^5 cells were plated onto 100-mm tissue culture dishes. Each plate was transiently transfected by the calcium phosphate method with 1 μg of pAdvantage (Promega) and 10 μg of pC1neo, or pC1neo with Krs-1 or Krs-2. After 48 hr, the cells remained untreated or were treated with STR. The cells were harvested and lysed as described for NIH 3T3 cells. Immunoprecipitations with the αHA antibody were carried out as described for the αKrs immunoprecipitations using 115 μg of 293 cell lysate. In-gel kinase assays and Western blot analyses were conducted as described using 20% of each immunoprecipitate. Immunoreactive protein was detected by enhanced chemiluminescence after probing the blot with 1 μg/ml αHA antibody and horseradish peroxidase-conjugated sheep α-mouse antibody.

RESULTS

NIH 3T3 cells were treated with a variety of agents, and alterations in kinase activity were analyzed by an MBP in-gel kinase assay (Fig. 1). A 63-kDa protein kinase activity was stimulated by STR and OA as well as by high concentrations of sodium *m*-arsenite and extreme heat shock at 55°C. Stress induced by reagents such as cycloheximide, carbonyl cyanide *m*-chlorophenylhydrazone, heat shock at 45°C, hydrogen peroxide, osmotic stress, and ultraviolet irradiation failed to induce p63 activity (Fig. 1, data not shown). STR treatment induced cell death morphologically consistent with apoptosis in all cell types examined; however, treatment and subsequent death induced by fatal stimuli such as TNFα or anti-Fas antibody failed to activate p63 in NIH 3T3 cells, L929 cells, and A673 cells (ref. 30, data not shown). Contrary to our expectations for a Src-activated kinase, growth-promoting stimuli such as epidermal growth factor, serum, and phorbol esters also failed to stimulate p63 activity (H.-C.R.W. *et al,* unpublished data). These data indicate that p63 is sensitive to specific cellular stress.

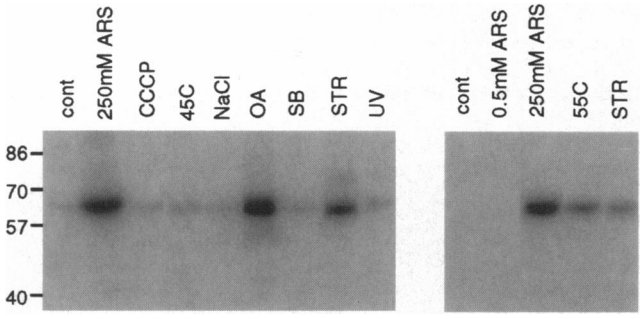

FIG. 1. p63 activity following stress. NIH 3T3 cells remained untreated or were treated with sodium arsenite (ARS), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), heat shocked at 45°C or 55°C, osmotically stressed with NaCl or sorbitol (Sb), treated with OA, STR, or UV-irradiated. An MBP in-gel kinase assay was conducted using 20 μg of soluble protein per sample, and the gel was subjected to autoradiography.

As in NIH 3T3 cells, STR-stimulated p63 activity in murine T-cell hybridomas, and these cells were used for purification of the protein kinase (Fig. 2*A*). The 63-kDa activity was followed through the indicated chromatography steps by the MBP in-gel kinase assay. Coincident elution of distinct Coomassie blue-stained proteins and in-gel kinase activities of 61 kDa and 63 kDa was noted following fractionation on the final Mono Q column (Fig. 2). The 61-kDa protein kinase scores poorly in the in-gel assay compared with the 63-kDa activity; therefore, the 61-kDa activity was not noted until this final purification step.

Peptide sequence was obtained from both the 61-kDa and 63-kDa species, and a 481-bp product was generated by PCR with oligonucleotides corresponding to a peptide from the p61 sequence and the predicted sequence from a partial cDNA

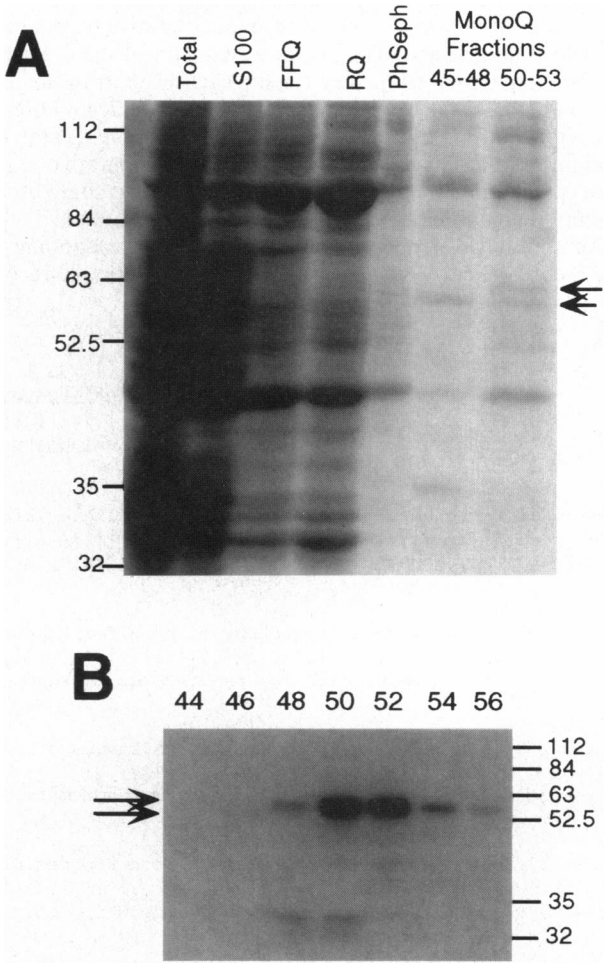

FIG. 2. Purification of p63. (*A*) The p63 activity was purified from STR-treated murine T-cell hybridomas. Aliquots were saved from the total lysate (0.004%), supernatant from the ultracentrifugation (S100, 0.006%), and the pools of p61/p63 activity from the Fast Flow Q column (FFQ, 0.015%), Resource Q column (RQ, 0.03%), phenyl Sepharose (PhSeph, 0.125%), and Mono Q columns (0.5%), combined with Laemmli sample buffer, and fractionated on an SDS/9% polyacrylamide gel. The gel was then stained with Coomassie blue to visualize the proteins. Arrows indicate the positions of the 61-kDa and 63-kDa proteins. (*B*) Two percent of each of the indicated fractions that eluted from the Mono Q anion exchange column were combined with Laemmli sample buffer and applied to an SDS/10% polyacrylamide gel containing 0.4 mg/ml copolymerized MBP. The gel was processed for an in-gel kinase assay and subjected to autoradiography.

previously deposited in GenBank (40). This PCR product was used as a probe to screen 1×10^6 plaques from a human Jurkat cDNA library, and 50 positives were detected. The eight clones of greatest intensity were further analyzed, and sequence analysis indicated that these clones fell into two classes. Two clones contained a cDNA fragment that encoded peptides found in p61, whereas the others contained cDNAs that encoded predicted peptides from p63.

Sequencing of these cDNAs indicated that the clones encoded two distinct but closely related protein kinases with predicted molecular weights of 56.3 kDa and 55.6 kDa (Fig. 3*A*). During our functional analysis of the products of these cDNAs, the sequences of these proteins were reported by another group (33, 34). Because of the physiological behavior of these enzymes, we propose to denote p63 as *k*inase *r*egulated by *s*tress (Krs)-1 and p61 as Krs-2. The predicted initiating methionines for both proteins are favorable, based upon the Kozak rule, and the translation of each protein is terminated at an in-frame stop codon (42). Krs-1 and Krs-2 are 94% identical in the catalytic domain and 78% identical overall, and the acidic structure of the C-terminal putative regulatory domain is maintained. The charged character of the C terminus is likely to be responsible for the slightly aberrant migration on SDS/PAGE. Krs-1 and Krs-2 share significant homology with kinases of Ste20p family throughout their catalytic domains and have short acidic regions in their putative regulatory domains, similar to a region in Ste20p and some other Ste20p family members (Fig. 3*B* and data not shown).

An antibody was raised against a peptide corresponding to residues 301–322 in Krs-2 (Fig. 3*A*), and this antibody recognized two proteins of 61 kDa and 63 kDa in murine T-cell hybridomas by Western blot analysis (Fig. 4*A*). This antibody was used to immunoprecipitate Krs-1 and Krs-2 from untreated and STR-treated hybridomas, and kinase activity was scored by the in-gel assay. Both Krs-1 and Krs-2 activities were immunoprecipitated from hybridomas. The activity of both kinases was stimulated by STR, and immunoprecipitation of these activities was competed with immunizing peptide (Fig. 4*B*). The immunoprecipitates were greatly enriched in Krs-2 as the antibody appears to preferentially recognize Krs-2 in immunoprecipitations due to sequence divergence between Krs-1 and Krs-2 within the immunizing peptide (Fig. 4*A*). As a result, both Krs-1 and Krs-2 activities were detected in in-gel kinase assays of immunoprecipitates, whereas only Krs-1 activity was evident after analyzing cell lysates directly.

The cDNAs encoding HA-tagged Krs-1 and Krs-2 were transiently transfected into 293 cells, and the transfectants remained untreated or were treated with STR. HA-tagged Krs-1 and Krs-2 were immunoprecipitated from the transfected cells, and the resulting kinase activity was scored with the MBP in-gel kinase assay. An STR-stimulated kinase activity was detected in the immunoprecipitates from Krs-1- and Krs-2-transfected cells (Fig. 5). These data indicate that the protein kinases encoded by the Krs cDNA clones are stimulated by STR as are the originally identified 61-kDa and 63-kDa activities.

DISCUSSION

We report here the characterization of a 63-kDa protein kinase activity that is stimulated by extreme forms of stress such as

A

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                                                     1                                                  2
Krs-1   1 ...meqppapksklkklsedsltkqpeevfdvleklqegsygsvfkaihkesgqvvaikqvpvesdlqeiikeisimqqcdspyvvkyygsyfkntdlwi 97
             :: . :|:..||||.||||||||||||||||||||||||:||||||.||:|||||||||||||||||||||||||||||.||||||||||||||||
Krs-2   1 metvqlrnpprrqlkkldedsltkqpeevfdvleklqegsygsvykaihketgqivaikqvpvesdlqeiikeisimqqcdsphvvkyygsyfkntdlwi 100
                                                     1                                          2

Krs-1  98 vmeycgagsvsdiirlrnktliedeiatilkstlkgleylhfmrkihrdikagnillnteghakladfgvagqltdtmakrntvigtpfwmapeviqeig 197
          |||||||||||||||||||.|||||||.||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Krs-2 101 vmeycgagsvsdiirlrnktltedeiatilqstlkgleylhfmrkihrdikagnillnteghakladfgvagqltdtmakrntvigtpfwmapeviqeig 200
                             3            4

Krs-1 198 yncvadiwslgitsiemaegkppyadihpmraifmiptnppptfrkpelwsddftdfvkkclvknpeqratatqllqhpfiknakpvsilrdliteamei 297
          |||||||||||||.|||||||||||||||||||||||||||||||||||||||:||||||.||||.|||||||||||||||||::.||.||||||||.|||::
Krs-2 201 yncvadiwslgitaiemaegkppyadihpmraifmiptnppptfrkpelwsdnftdfvkqclvkspeqratatqllqhpfvrsakgvsilrdlineamdv 300

Krs-1 298 kakrheeqqrele.eeeensdedeldshtmvktsvesvgtmratstmsegaqtmiehnstmlesdlgtmvinsedeeeedgtmkrnatspqvqrpsfmdy 396
          | ||:|.||||:: ::||||:|||:|| |||:. .::||:|..|||.:||.|||||:.| |.|:|||||||.|||||| |||||.... |..:|||::|
Krs-2 301 klkrqesqqrevdqddeenseedemdsgtmvravgdemgtvrvastmtdgantmiehddt.lpsqlgtmvinaedeeee.gtmkrrdetmqpakpsfley 398

                                                                                                   3
Krs-1 397 fdkqdfknkshencnqnmhepfpmsknvfpdnwkvpqdgdfdflknlsleelqmrlkaldpmmereieelrqrytakrqpildamdakkrrqqnf 491
          |:..: |:.  :. ...:.:|:. |      .:||:|||||::|||.:.:|:||.|| |||||||.||||:||:| .||||||||::|||||||||
Krs-2 399 feqke.kenqinsfgksvpgplkns.....sdwkipqdgdyeflkswtvedlqkrllaldpmmeqeieeirqkyqskrqpildaieakkrrqqnf 487
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B

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Krs-1    306   Q Q R E L E . E E E E N S D E D E L D S H T   326
               : . . : . :   : : : : : . | : : . : : .   .
Ste20p   895   E N M D A D E D N D D D N D N E H I N K T N   917
               : . . . : . | : | : : : : : . : : : . : : . . .
Krs-2    308   Q Q R E V D Q D D E E N S E E D E M D S G T   329
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FIG. 3. Comparison of Krs-1 and Krs-2. (*A*) The amino acid sequences of Krs-1 and Krs-2 were aligned using the GAP program from the Genetics Computer Group (Madison, WI). The tryptic peptides obtained from purified Krs-1 and Krs-2 are underlined and numbered. The peptide used to make the antibody is indicated in bold type. The sequences of these cDNAs have been reported (34, 35). (*B*) An acidic region of Krs-1 and Krs-2 is aligned with a similar region in Ste20p. All alignments were generated using software from the Genetics Computer Group with gap weights set at 3.0 and gap length weights at 0.1. Identical amino acids are indicated by "|", and conserved amino acids are indicated as ":" and ".", according to the described programs (41).

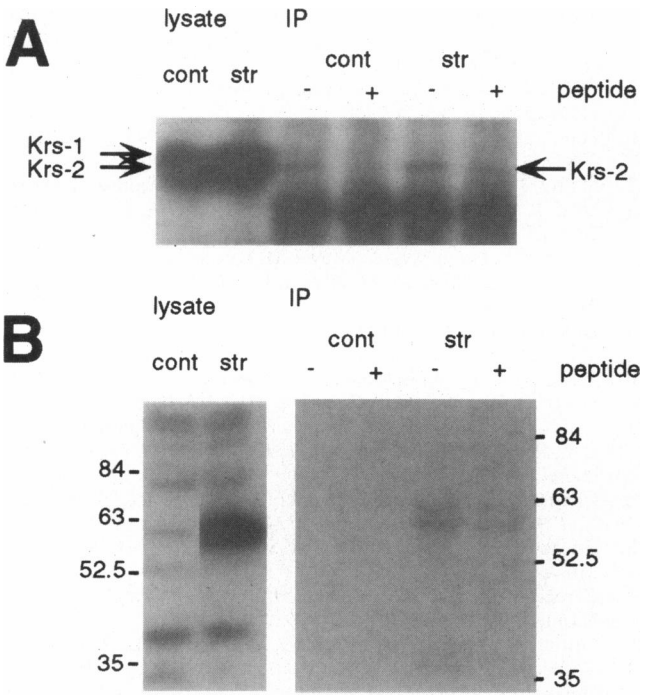

FIG. 4. The αKrs antibody recognizes STR stimulated protein kinases in T-cell hybridomas. Cell lysates were collected from untreated and STR-treated T-cell hybridomas. (*A*) The lysates were either fractionated by SDS/PAGE or immunoprecipitated with αKrs antibody initially then fractionated by SDS/9% polyacrylamide gel. The gel was transferred to Immobilon-P and processed for Western blot analysis with the αKrs antibody. Where indicated, the αKrs antibody was preincubated with 2 μg of the immunizing peptide before immunoprecipitation. (*B*) Cell lysates and immunoprecipitates were applied to a SDS/10% polyacrylamide gel containing 0.4 mg/ml copolymerized MBP. The gel was processed for an MBP in-gel kinase assay as described, except 0.5 μm unlabeled ATP and 25 μCi/ml [γ-^{32}P]ATP was used in the kinase assay.

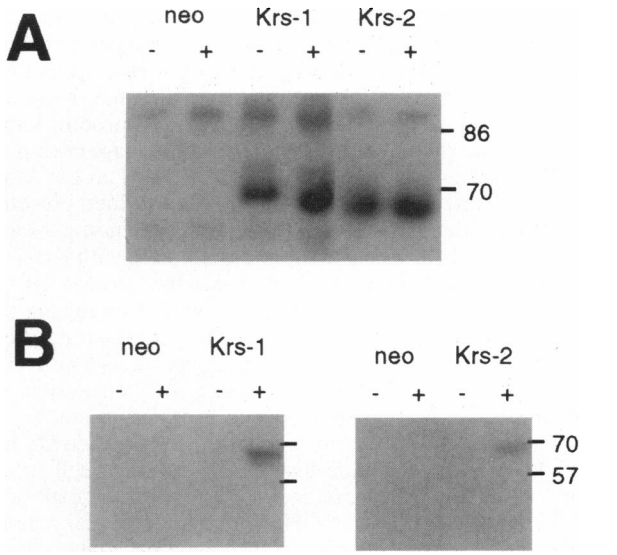

FIG. 5. Krs-1 and Krs-2 cDNAs encode STR-stimulated protein kinases. The cDNAs encoding Krs-1 and Krs-2 were epitope tagged with the HA epitope and transiently transfected into 293 cells with the pAdvantage construct. Soluble lysates were collected and subjected to immunoprecipitation with the αHA antibody. (*A*) Immunoprecipitates were fractionated by SDS/9% polyacrylamide gel. The gel was transferred to Immobilon-P and Western blot analysis was conducted using the αHA antibody. (*B*) The αHA immunoprecipitates were fractionated by SDS/10% polyacrylamide gel containing copolymerized MBP. The gel was processed for an in-gel kinase assay as described using 0.5 μm unlabeled ATP and 25 μCi/ml [γ-^{32}P]ATP. The gels were exposed to film for 2.5 hr (Krs-1) or 14 hr (Krs-2).

OA, heat shock, sodium arsenite, and STR. This protein kinase as well as another highly related, copurifying kinase were purified from T-cell hybridomas and denoted Krs-1 and Krs-2. Using peptide sequence information obtained from the purified proteins, we isolated putative cDNAs encoding these kinases. Precipitation of STR-stimulated protein kinase activities with an antibody recognizing Krs-1 and Krs-2 and STR activation of transiently overexpressed Krs-1 and Krs-2 indicates that the cDNAs encode the original 61-kDa and 63-kDa activities identified in the purification. It is likely that Krs-1 is equivalent to the Src-activated protein kinase originally observed in chicken embryo fibroblasts (30). Its delayed activation by Src and lack of regulation by growth-promoting stimuli suggest that Krs-1 might function in the late morphological alterations that result upon pp60$^{v\text{-}src}$ transformation, in addition to the pivotal role this kinase might play in the cellular response to extreme stress.

Sequence analysis demonstrated that these stress-regulated kinases are members of a subfamily and highly related to the Ste20p family within their catalytic domains. Others have identified these kinases by PCR cloning but do not suggest a physiological function (33, 34). Outside of their kinase domains, there are significant differences between members of this family. The fundamental organization of the kinases varies. Krs-1 and Krs-2, like germinal center kinase, Sps1p, and putative *Caenorhabditis elegans* Ste20p family members, have N-terminal catalytic domains, whereas Ste20p and Paks have C-terminal catalytic domains. Additionally, Krs-1 and Krs-2 lack the Rac- and Cdc42-interacting domains and potential Src homology domain 3-binding domains present in some, but not all, Ste20p family members. However, the presumptive regulatory domains of Krs-1 and Krs-2 are not entirely dissimilar from their relatives. Krs-1 and Krs-2, like Ste20p, have a short acidic region in their noncatalytic domains (Fig. 3*B*). Other Ste20p family members, including mammalian Paks and putative *C. elegans* Ste20p homologs, also have acidic regions 10–15 aa in length. The possible influence these short regions may have upon characteristics such as subcellular localization or substrate recognition remains to be examined.

As is the case for initial events in the activation of Jnk and p38, the molecular mechanisms responsible for Krs activation are unclear. In the pheromone-responsive pathway in *S. cerevisiae*, *STE20* is genetically downstream of both the rho-related *CDC42* and the genes encoding the $\beta\gamma$ subunits of a heterotrimeric G protein (9, 10, 43, 44). Moreover, Pak1 was identified based upon its ability to interact with the mammalian G proteins Cdc42 and Rac, and this interaction results in increased Pak activity (31). Germinal center kinase has recently been shown to interact with the Ras-related G protein, Rab8 (45). These data suggest that members of the Krs subfamily are likely to lie immediately downstream of a Ras-related or heterotrimeric G protein. However, preliminary data indicate that Krs-1 does not interact with recombinant glutathione *S*-transferase-tagged Cdc42, Rac, or Rho (unpublished results), nor do these kinases contain an obvious pleckstrin homology domain through which they might interact with heterotrimeric G proteins (46). The activity of many protein kinases is often regulated by phosphorylation. The ability of both a kinase inhibitor (STR) and a phosphatase inhibitor (OA) to increase Krs-1 activity suggests that Krs-1 activity may be regulated either directly or indirectly by phosphorylation. Moreover, it has been reported that the activity of overexpressed Krs-2 could be modestly increased with phosphatase treatment (33). The role of phosphorylation in regulating an activated form of the Krs subfamily remains to be examined, however.

The close similarity between the catalytic domains of Krs-1, Krs-2, and other Ste20p family members suggests that this novel kinase subfamily functions at a very early step in a Map kinase pathway. Our observations indicate that Krs-1 and Krs-2 associate with another STR-stimulated protein kinase, suggesting that this subfamily functions in a kinase cascade. In the purification of Krs-1 and Krs-2, a third in-gel kinase activity and corresponding Coomassie blue-stained protein of ≈40 kDa was detected (Fig. 2*B*). In addition, an in-gel kinase activity of ≈40 kDa was coimmunoprecipitated with Krs-1 and Krs-2 from STR stimulated murine T-cell hybridomas, and this coprecipitating activity was competed with immunizing peptide (Fig. 4*B*). Determining the identity of this associated kinase activity as well as other interacting proteins may provide important clues to aid in elucidating the regulation and function of this unusual subfamily of protein kinases.

The stimuli identified thus far that induce Krs activity have common phenotypical ramifications; all reduce cell attachment and result in eventual death, with the exception of transformation by $pp60^{v\text{-}src}$. However, the cellular changes induced as a result of v-src transformation make chicken embryo fibroblasts susceptible to induction of apoptosis by some nonsteroidal anti-inflammatory drugs (47). Activation of the Krs subfamily is not a common response in dying cells as evidenced by the inability of TNFα, under both apoptotic and necrotic conditions, and anti-Fas antibodies to stimulate Krs-1 (data not shown). Elucidation of the event(s) leading to the activation of Krs-1 and 2 and their potential function in responding to or effecting cytoskeletal dynamics are now necessary.

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