Human Tribbles, a Protein Family Controlling Mitogen-activated Protein Kinase Cascades

Control of mitogen-activated protein kinase (MAPK) cascades is central to regulation of many cellular responses. We describe here human tribbles homologues (Httrbs) that control MAPK activity. MAPK kinases interact with Trbs and regulate their steady state levels. Further, Trbs selectively regulate the activation of extracellular signal-regulated kinases, c-Jun NH2-terminal kinases, and p38 MAPK with different relative levels of activity for the three classes of MAPK observed depending on the level of Trb expression. These results suggest that Trbs control both the extent and the specificity of MAPK kinase activation of MAPK.

Mitogen-activated protein kinase (MAPK) cascades control the activity of three sets of effector protein kinases (extracellular signal-regulated protein kinases (ERKs), Jun kinases (JNKs), and p38s). The central element in each MAPK pathway is a module of three protein kinases, MAPKK kinase, MAPK, and MAPK (1). The three sets of effector MAPK differ in type of activating stimulus: JNKs and p38/HOG-1 primarily respond to stress (e.g. heat shock), and ERKs primarily respond to mitogens. However, a stimulus can activate more than one class of MAPK; the contribution of each pathway is cell type-dependent, and MAPK pathways can both synergize and antagonize. This is caused in part by regulatory proteins influencing signaling by a range of mechanisms including scaffolding (e.g. JIP-1, STE5), regulating localization (e.g. Kar), or recruitment to targets (e.g. 14-3-3 proteins) (2–4). Here we describe a novel family of MAPK control proteins, homologues of Drosophila tribbles.

Drosophila tribbles was shown to regulate String activity and hence mitosis during ventral furrow formation (5–8). A canine Trb-2-like protein has been described in the literature as a transiently expressed, mitogen induced, and highly labile cytoplasmic phosphoprotein, but its biological function was not characterized (9, 10). Rat Trb was shown to be rapidly up-regulated during neuronal cell apoptosis (11). Recently Trb-3 has been reported to regulate Akt activation in liver by insulin (12) and regulate ATF4 activity (13, 14). We show here that Trbs bind to MAPKK and regulate MAPK activation suggesting that Trb function may be broader than reported previously.

MATERIALS AND METHODS

Plasmids—FLAG-MK7 (15), FLAG-MEK-1 (16), IL-8-luc (17), and LHRE-TK-luc (18) were described earlier. VI2 Ras was a gift of J. Downward. pAP-1 luc, pNF-xB luc, pFR luc, pFA-CHOP, pFA2-Erk-1, pMEKK-1, pMEK-1, and pMEK-3 were part of the PathDetect system (Stratagene). Quantitative real time-PCR was performed to characterize the expression profile of human tribbles genes by using the Human Rapid-Scan panel (Origene). MRNA levels are expressed as relative units normalized for glyceraldehyde-3-phosphate dehydrogenase expression.

Cell Cultures and Transfections—HeLa (ECACC, 85060701) and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and penicillin-streptomycin. Raw cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin-streptomycin. Cells (1.5 x 10⁴ per well) were seeded into 96-well tissue culture plates 24 h prior to transfection. Transfections were performed using SuperFect (Qiagen) according to the manufacturer’s advice; each well received 500 ng of inducible reporter construct (pIL-8 luc, pAP-1 luc, pNF-xB luc, or pLHRE-TK luc), 100 ng of pTK-RLuc (Promega) for normalization of transfection efficiency, and 50 ng of htrb-1 or htrb-3 expression vectors under investigation unless stated otherwise in the appropriate figure legend. 500 ng of pFR luc, 100 ng of pTK-RLuc, and 10 ng of pFA-CHOP or pFA2-Erk-1, 25 ng pMEK-1, or pMEK-3 plasmids were transfected to specifically activate p80 or ERK and to study the effect of htrb-3 on the activation. Sufficient pCDNA3.1 (Invitrogen) (“empty vector”) was added to keep the total DNA dose constant at 700 ng/well. 2 h after transfection, cells were washed, and 100 µl of fresh medium was added. Triplicate wells were transfected for each treatment. Stimulations were performed for 4 h (unless indicated otherwise) 24 h later. 2 ml IL-1α or 10 ng/ml tumor necrosis factor α, 0.5 µg/ml human growth hormone, 50 ng/ml PMA, or 10 ng of the other cytokines listed on Supplementary Table I was used (unless stated otherwise on the figure). Agonists were prepared and added as 10x stocks in 11 µl of phosphate-buffered saline. Reporter levels were measured following 4 h of stimulation using the dual-luciferase system (Promega) as recommended by the manufacturer.

Cytokines—IL-β was a kind gift from the ImmuneX Corporation. The other human cytokine preparations were kindly provided by Dr. Steve Poole, National Institute for Biological Standards Control.

Western Blotting—For detection of pJun, pERK, and pMKK4, poly-
clonal antibodies were purchased from Sigma. Protein concentrations of cell lysates were determined, and an equal amount of total protein was loaded in each lane. Kinase assays were performed by using the appropriate kits from New England Biolabs.

RESULTS

Human tribbles Identification and Sequence—Using a transcription expression screen for gene products mediating inflammatory cytokine signaling (19–21), we identified a human tribbles homologue (htrb-1) which regulated the human IL-8 promoter in HeLa cells. The library clone encoded a portion of the 3′-untranslated region extending to the poly(A) tail (the library was oligo(dT)-primed) in the expression vector (pCDM8) in the sense orientation. PCR experiments using coding region primers to detect endogenous trb-1 message but not the truncated noncoding library-derived transcript showed that transfection of the library clone into HeLa cells caused a >10-fold increase in the level of endogenous trb-1 message, which correlated with inhibition of IL-8 promoter activity. Two other human tribbles homologues, htrb-2 and htrb-3 (41 and 51% identical to Htrb-1, respectively), were identified by database searching; sequences have been submitted to GenBankTM (accession numbers AF250310 and AF250311).

A family comprised of three trbs is found in mammals, and orthologues have been identified in both vertebrates and invertebrates. All three Trb proteins share a central Trb domain. In addition, each has N-terminal (70–100 residues) and C-terminal (~25 residues) domains, which are neither closely related to any other sequences nor closely related to each other. The Trb domain is homologous to the protein serine-threonine kinases but lacks the active site lysine and is predicted to be kinase dead as shown for canine Trb-2 and Tribbles (5, 9).

Quantitative real-time-PCR experiments showed that trb-1 mRNA was expressed in most human tissues with the highest levels in skeletal muscle, thyroid gland, pancreas, peripheral blood leukocytes, and bone marrow (Supplementary Fig. 1). trb-2 levels were highest in peripheral blood leukocytes, and trb-3 levels were highest in pancreas peripheral blood leukocytes and bone marrow; in addition HeLa cells were found to express trbs 1–3. (Supplementary Fig. 1).

Trbs Regulate AP-1—When overexpressed, Htrb-1 repressed basal activity of the NFκB and AP-1-regulated IL-8 promoter in HeLa cells in culture but not its activation by IL-1 and tumor necrosis factor α (Fig. 1A). The NFκB sites mediate cytokine activation, whereas the AP-1 sites control basal activity (22). Our results therefore suggest that Trb-1 acts selectively on pathways leading to AP-1. Consistent with this, testing specific NFκB or AP-1 reporters activated by MEKK-1 co-transfection showed that Htrb-1 inhibited AP-1 activity but not NFκB (Fig. 1, B and C) nor a signal transducers and activators of transcription-responsive LHRE promoter (Fig. 1D). Specificity was also analyzed by testing a panel of human cytokines in the presence or absence of Htrb-3; AP-1 activation was inhibited (Fig. 1H), whereas NFκB activation was not (Supplementary Table 1). Thus, whether activated by overexpression of upstream components or by physiological agonists, pathways leading to AP-1 activation were specifically inhibited by Trb overexpression.

We have shown above that up-regulation of endogenous Trb-1 and ectopic overexpression of Trb-1 and Trb-3 inhibited AP-1 activity in HeLa cells. Next we examined the effects of suppressing endogenous Trb expression. An antisense construct encoding the htrb-3 5′-untranslated region and N-terminal domain was co-transfected with AP-1 or NFκB reporters using either MEKK-1 or NFκB inducing kinase as activators (Fig. 1, E–F). Trb-3 mRNA expression detected by quantitative real-time-PCR using primers spanning the central Trb domain was reduced by >70% (Fig. 1G), whereas Trb-1 levels were only slightly altered by transfecting a high dose of AS-Trb-3 con-
Role of Human Tribbles in MAPK Signaling

Fig. 2. Htrb overexpression modulates the activation of Ras/MEK-1/ERK pathway. A, HeLa cells were transfected with an AP-1 reporter, a V12Ras expression construct, or an Htrb-1 or Htrb-3 expression construct, and reporter Luc was measured. B, HeLa cells transfected with empty vector (top), Htrb-1 vector (middle), or Htrb-3 vector (bottom) were stimulated by PMA, and activated (pERK) and total (apo) ERK levels were assayed by Western blotting. C, HeLa cells were transfected by the stated dose of Trb-1 or Trb-3 expression plasmid, stimulated with PMA, and activated and total ERK levels were assayed by Western blotting. Lane 1, 10 μg of carrier DNA only; Lane 2, 50 ng of Trb1; Lane 3, 200 ng of Trb1; Lane 4, 1 μg of Trb1; Lane 5, 4 μg of Trb1; Lane 6, 50 ng of Trb3; Lane 7, 200 ng of Trb3; Lane 8, 1 μg of Trb3; Lane 9, 4 μg of Trb3. D, ERK kinase activity was measured on the same extracts in an in vitro kinase assay using recombinant Elk as a substrate.

Trbs Regulate ERKs—Because Trbs are mitogen-induced and regulate String/cdc25, we examined the effect of Trb levels on the control of the Ras/MEK/ERK module. V12Ras-driven AP-1 activation was blocked by overexpression of both Trb-1 and Trb-3 (Fig. 2A). Also, HeLa cells were stimulated with PMA, and phospho-ERK levels were determined by Western blotting; basal levels were increased by Htrb-1 or Htrb-3 (Fig. 2B), correlated with enhanced ERK activity measured in an in vitro kinase assay (Fig. 2D). Although expression levels of Trb-1 and Trb-3 were comparable (see Fig. 4), ERK activation was enhanced only using low doses of Trb-3 (Fig. 2C), and ERK potentiation was enhanced in the presence of all doses of Trb-1 within the range tested (Fig. 2C), suggesting that the system is more sensitive toward Trb-3 levels. Comparison between constitutive (transfection, Fig. 2A) and transient (PMA treatment, Fig. 2, B–D) stimuli showed that Trbs are capable of either up- or down-regulating MAPK activity.

MAPKK Interacts with Trbs—Overexpression of htrb-1 inhibited MEKK-1 mediated AP-1 but not NFκB activity (Fig. 1, E and F). A similar effect was seen when an AP-1 reporter was stimulated by PMA (Fig. 3A). To characterize the possible site of Htrb action, we tested whether increasing the dose of PMA (Fig. 3A) or MEKK-1 (Fig. 3B) could override the effect of Htrb-3. This was not the case, suggesting that htrb-3 controls a rate-limiting step downstream of MEKK-1. MEKK-1 phosphorylates MKK7 and/or MKK4, and activation of these kinases leads (via JNKs) to phosphorylation of transcription factors including c-Jun and CREB2. Western blotting showed that both the kinetics and the extent (as judged by gel mobility) of PMA-induced jun phosphorylation were altered by Trb-3 (Fig. 3C). Thus the locus of action of trbs in the JNK pathway is at or downstream of MAPKKs and at or upstream of c-jun. p38 MAPK was not activated by PMA in this system, or downstream of MAPKKs and at or upstream of c-jun. p38 MAPK was not activated by PMA in this system, or downstream of MAPKKs and at or upstream of c-jun. p38. At low doses, Htrb-3 enhanced JNK and ERK activation (with a more pronounced effect on ERKs) but inhibited p38 (Fig. 5A). At higher doses the activation of all three pathways was inhibited relative to control. Thus there appears to be a different optimal set point for each pathway (Fig. 5A), suggesting that Htrbs may regulate relative activation of the three classes of MAPK.

DISCUSSION

We show that Trbs appear necessary for MAP kinase pathway function and are inhibitory at high levels. Several mechanisms can account for these findings. For example, Trbs might be scaffolds, both over- and underexpression of which has been shown to inhibit MAPK signaling (23–26).

Our data show that MAPKKs not only bind Trbs but also appear to stabilize them. Further, Trb proteins have been reported to be rapidly mitogen induced and to have short half-lives (9, 10), and we have found that Trb mRNAs are also rapidly induced by mitogens and have short half-lives.3 Taken together, these data suggest that Trb/MAPK interactions are dynamic and regulated by external signals. In addition, MAPK...
cascades have been suggested to incorporate a positive feedback loop leading to nonlinear behavior (24). Modeling using ordinary differential equations shows that with these elements in place, Trb proteins could act either as activators or as inhibitors of MAPK activity, depending on the ratio of Trb to MAPKK levels in the cell.4 Similar observations have been made by us and others for the NFκB (27, 28) and the cell cycle control systems (29). We also note that these systems are capable of producing much more complex behaviors than the simple nonmonotonic dose responses we report here, for example the oscillations observed in cells from IκB knock-out mice and reproduced in simple mathematical models (28).

4 S. K. Dower and E. Kiss-Toth, unpublished data.
be an Akt inhibitor, UAS-driven Trb overexpression causes G2 mitotic arrest in imaginal disk cells, and the original \( \text{trb} \) clone we detected in our screen was active by virtue of its capacity to modulate endogenous Trb expression. Fly tribbles regulates cell cycle progression, blocking String action by promoting its degradation. Thus \( \text{trb} \) flies show defective gastrulation because of elevated zygotic String activity driving premature mitosis (5, 7, 8). However, these experiments did not identify a molecular mechanism of tribbles action. A large body of literature shows MAPK pathways to be regulators of String/cdc25 (30–35). We speculate that tribbles may also regulate MAPKK activity.

There are apparent discrepancies between our results and those reported for \textit{Drosophila} tribbles. First we observed no effect on cell cycle progression in HeLa cells caused by either overexpression or repression (with antisense) of either \( \text{Trb-1} \) or \( \text{Trb-3} \). This may be because HeLa cells express all three \( \text{Trbs} \), and the two tested here are partially functionally redundant. We also found that both overexpression and repression of expression of \( \text{Trbs} \) inhibited AP-1 activity and that whereas \( \text{Trb-3} \) overexpression inhibited the activity of all three MAPK cascades relative to control, at intermediate levels a modest enhancement of JNK and ERK activity was observed. However, overexpression of String and/or tribbles in \textit{Drosophila} appears to have opposite effects on mitosis in cytokocytes during oogenesis and in the ventral region during embryogenesis, an effect suggested to be caused by changes in the duration of G2 resulting from perturbations in the relative levels of String and Trb (8). Further, it has been reported that tribbles action in \textit{Drosophila} is cell type-specific (7). We speculate that this may well apply for the mammalian homologues as well. In line with this model, we have found that MEKK1-mediated AP-1 activation is blocked by various tribbles in a cell type-dependent manner (Fig. 5B).

A recent report shows that Trb-3 interacts with Akt and that elevated Trb levels block Akt activation in the liver (12). Our current understanding of Akt signaling cascades places this protein kinase at the level of MAPKKs in the “signaling hierarchy” (Fig. 5C), suggesting that the observations of Du \textit{et al.} (12) are consistent with a broader picture of Trb function as regulators of intermediate steps in these protein kinase cascades. In conclusion, the data in this report suggest that mammalian Trbs bind to and regulate MAPKK and may thus control the relative activation of MAPK by incoming signals.

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Fig. 5. \textit{MAPK activation is modulated by \textit{trb} expression.} A, to determine the activation of terminal MAPKs the PathDetect (Stratagene) system was used. pFR luc reporter was transfected into HeLa cells together with pFA-CHOP, activated by pMEK-1 (p38) (\( \text{p38} \)), or with pFA2-Elik-1, activated by pMEK-1 (ERK) (\( \text{ERK} \)); AP-1 luc was stimulated by cotransfection with pMEK-1 (JNK) (\( \text{JNK} \)). B, to assess the cell type specificity of Trb action, HeLa, NIH 3T3, and RAW 264.7 cells were transiently transfected with AP-1 reporter, MEKK-1 expression vector, and \( \text{hrb-1} \) or \( \text{hrb-3} \) expression constructs. C, the proposed site of \( \text{trb} \) action in MAPK cascades.
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