Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 343 (2006) 125–134
BBRC
www.elsevier.com/locate/ybbrc

The TNF receptor, RELT, binds SPAK and uses it to mediate p38 and JNK activation ☆

Tara C. Polek, Moshe Talpaz, Taly Spivak-Kroizman *

Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

Received 17 February 2006
Available online 2 March 2006

Abstract

Receptor expressed in lymphoid tissues (RELT) is a new member of the TNFR family with little known regarding its signaling. Typically, TNFRs engage TRAFs for activation of NF-κB and MAPK cascades. We found that RELT does not use the standard signaling pathways characteristic of other TNFRs. While overexpression of RELT in 293 cells induced p38 and JNK activation, it did not activate NF-κB. In addition, no binding of RELT to TRAF1,2,3,5, or 6 was detected. Using a yeast two-hybrid system, we identified a Ste20-related proline-alanine-rich kinase (SPAK) that binds RELT. Disruption of the SPAK binding motif, ^{349}RFRV, in RELT inhibited RELT activation of p38 and JNK. In addition, a kinase-dead SPAK acted as an inhibitor of RELT signaling. Thus, we conclude that RELT does not rely on the canonical TRAF pathways for its function, but instead uses a kinase, SPAK, to mediate p38 and JNK activation.

Keywords: Signal transduction; TNF receptors; Kinases; SPAK; TRAFs

Members of the tumor necrosis factor receptor (TNFR) family are important modulators of biological processes including immune responses, hematopoiesis, and tumor suppression [1,2]. The extracellular domains of TNFRs have one to four distinct cysteine-rich repeats that are evolutionarily conserved and serve as a hallmark for the classification of new receptors as members of the TNFR family [3]. TNFRs are further sorted into three groups based on their intracellular structural attributes: death domain (DD)-containing receptors, TRAF-binding receptors, and decoy receptors [2]. DD-containing receptors typically induce apoptosis by recruiting adaptors with DDs that lead to the activation of caspase cascades. In contrast, TRAF-binding receptors, which lack DDs, often govern cellular proliferation and differentiation, utilizing TRAFs to activate downstream signaling pathways, including NF-κB and MAPK pathways (p38, JNK, and ERK). Decoy receptors, which are either soluble, secreted receptors or receptors with truncated cytoplasmic domains, function by competing with their cognate receptors for ligand binding, thus inhibiting their signaling.

RELT is a newly identified TNFR, that currently has no known ligand and whose mRNA is highly expressed in various hematologic tissues and cell lines [4]. RELT was cloned by searching the human expressed sequence-tagged database for novel molecules homologous to the extracellular domain of OX40 [4]. It has two cysteine-rich domains (one complete and the other incomplete) in its extracellular domain and does not contain a cytoplasmic DD. Overexpression of RELT has been shown to activate NF-κB and bind to TRAF1. The observation that RELT binds only to TRAF1, but not TRAFs 2,3,5 or 6, was intriguing and unusual for TNFRs. We therefore sought to elucidate the downstream signaling pathways activated by RELT.

☆ DNA sequencing was performed in the DNA Analysis Core facility at the M.D. Anderson Cancer Center, which is supported by National Cancer Center Grant CA-16672 (DAF).
* Corresponding author. Fax: +1 713 792 5963.
E-mail address: tspivakk@mdanderson.org (T. Spivak-Kroizman).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.02.125

We found that RELT induced activation of the p38 and JNK, but not NF-κB, pathways. It did not bind any of the TRAF adaptor molecules directly, but instead bound to the kinase SPAK. The binding of SPAK to RELT was direct and mapped to residue F350 within RELT's cytoplasmic domain. In addition, SPAK played a role in mediating RELT-induced activation of the p38 and JNK MAPK cascades. Our study establishes RELT as the first identified non-DD TNFR that does not bind TRAFs directly, but instead, binds to a kinase, SPAK. As a result, RELT does not activate NF-κB, but stimulates the p38 and JNK pathways via SPAK.

Experimental procedures

Chemicals and reagents. GST-Jun and GST-ATF2 from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Jun, anti-p38, and anti-phospho-ATF2 polyclonal antibodies were from Cell Signaling Technology, anti-FLAG antibodies were from Sigma–Aldrich, anti-Myc and anti-JNK1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HA tag monoclonal antibodies (12CA5) were from Roche Applied Science (Indianapolis, IN).

Construct generation and fusion protein purification. FLAG-RELT was constructed into the *Hin*dIII/*Bam*HI sites of pCMVFLAG1 by PCR amplification of amino acids 27–430 (without the signal peptide encoded within amino acids 1–26) from IMAGE clone #4873951 (GenBank BC017279, Invitrogen). GST-RELT$_{CD}$ was cloned by inserting amino acids 188–430 of the RELT cytoplasmic domain into the *Xba*I/*Sal*I sites of pGEX-KG. To construct the chimeric receptor RANK/RELT, amino acids 191–430 of RELT were fused to pCMVFLAG1-RANK241 [5]. GST-RANK$_{CD}$ was cloned as described previously [5].

Human TRAF1 and TRAF2 (IMAGE clones #3832475 (GenBank BC024145) and #6528158 (GenBank BC064662), respectively) and human TRAF3, a generous gift of Gail Bishop, Ph.D. (University of Iowa, Iowa City, Iowa) were cloned into the *Not*I/*Eco*RV sites of pcDNA3.1/*myc*-His(−) A. Mouse TRAF5 and TRAF6 were cloned into pCR3FLAG [5].

Full-length mouse SPAK (IMAGE clone #6843981 (GenBank BC064443)) was cloned into the *Nhe*I/*Bam*HI sites of pcDNA3.1/*myc*-His(−) A and into the *Eco*RI/*Xho*I sites of pGEX-KG. FLAG-SPAK-Myc/His (an N-terminal FLAG- and C-terminal Myc/His-tagged mouse SPAK) was created by subcloning SPAK-Myc/His into pCR3FLAG. Human oxidative stress responsive 1 (OSR1) was cloned from IMAGE clone #3163379 (GenBank BC008726) into the *Bam*HI/*Hin*dIII sites of pcDNA3.1/*myc*-His(−) A. Amino acids 1–260 of human Na–K–2C1 cotransporter 1 (NKCC1) were cloned from IMAGE clone #4824556 (GenBank BC033003) into pGEX-KG.

RELT(F/A), SPAK(K/E) [6], and SPAK(T/E) [7] mutants were generated by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

GST fusion proteins were purified essentially as described in [8] except that expression of GST-RANK was induced at 15 °C and buffers were at pH 7.4.

Transfections, immunoprecipitations, and Western blot analyses. 293 cells were transfected using calcium phosphate (Invitrogen Corporation) and HeLa cells using FuGENE 6 (Roche Applied Science). Cells lysis, immunoprecipitations, and Western blot analysis were performed as described in [8].

In vitro kinase assay. GST-fusion proteins were washed twice in low salt buffer (20 mM Hepes, 25 mM NaCl, and 1 mM DTT), incubated for 30 min at 30 °C with 12 μl of kinase buffer (50 mM Hepes, 20 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 mM DTT) containing 10 μCi [γ-^{32}P]ATP and 2 μg of the indicated substrate, and then analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

Nonradioactive kinase assay. Cells were cotransfected with the indicated receptor and either HA-tagged p38 or JNK kinase and then lysed as described above. Anti-HA antibodies were used for immunoprecipitations and samples were washed two times in lysis buffer and two times in Hepes buffer (20 mM Hepes, pH 7.4; 25 mM NaCl; and 1 mM DTT with protease inhibitors). The kinase assay was performed in 50 mM Hepes, pH 7.4; 20 mM $MgCl_2$; 1 mM DTT; 0.33 mM ATP, and 1 μg of substrate (GST-ATF2 for p38 and GST-Jun for JNK) and incubated for 30 min at 30 °C. Samples were subjected to Western blot analysis.

Ras recruitment yeast two-hybrid screen. A modified Ras recruitment yeast two-hybrid screen [9] generous gift of Dr. Ami Aronheim (Technion, Israel Institute of Technology, Haifa, Israel) was performed using RELT$_{CD}$ as the bait. RELT$_{CD}$ (amino acids 188–430) was subcloned with an N-terminal glycine linker domain into the *Bam*HI site of pMet425MycRasBam. This vector, which contains a pMet425 promoter, allows expression of the bait only in the absence of methionine and was used to transform the CDC25-2α ura3, lys2, leu2, try1, hisΔ200, ade2-101 strain of *Saccharomyces cerevisiae*. A clone with high bait expression and negative methionine regulation (as determined by anti-Myc Western blot) was transformed with a CytoTrap® human thymus library (Stratagene). Library plasmids from positive colonies (those that grew at 37 °C on galactose, but not glucose, in the absence of methionine) were electroporated into DH5α-competent cells and sequenced. Binding to RELT was verified by in vitro translation in the presence of [^{35}S]methionine (TNT Quick-Coupled Transcription/Translation System, Promega, Madison, WI) and pull-down analysis with GST-RELT$_{CD}$.

Results

RELT overexpression activates MAP kinase pathways but not the NF-κB pathway

Overexpression of RELT in 293 cells revealed the presence of two bands, a 46-kDa full-length receptor and a 22-kDa truncated form, probably resulting from proteolytic cleavage of the extracellular domain (data not shown) [6]. To circumvent this cleavage of wild-type RELT, we made a chimeric receptor with the intracellular domain of RELT (residues 191–430) fused to the extracellular domain (amino acids 33–241) of receptor activator of NF-κB (RANK). This chimeric receptor, termed RANK/RELT, was highly expressed and not cleaved like the wild-type receptor, which facilitated studying RELT-induced signaling pathways.

Since little is known about signaling pathways downstream of RELT, we investigated the ability of RELT to activate the p38 and JNK MAPK pathways. To this end, RELT and RANK/RELT were overexpressed in 293 cells, and an in vitro kinase assay was performed with the appropriate substrate (GST-ATF2 for p38 and GST-Jun for JNK) (Fig. 1). Overexpression of RELT or RANK/RELT induced phosphorylation of c-Jun and ATF2 indicating activation of the p38 and JNK signaling cascades (Figs. 1A and B). In addition, since the p38s and JNKs are potent upstream activators of the activator protein-1 (AP-1) transcription factor, we tested the ability of RELT to initiate AP-1 transcriptional activity. Indeed, RELT and RANK/RELT induced a 2.5- to 4-fold induction of AP-1 activity in a luciferase reporter assay, providing additional support for RELT's ability to activate p38 and JNK (Fig. 1C).

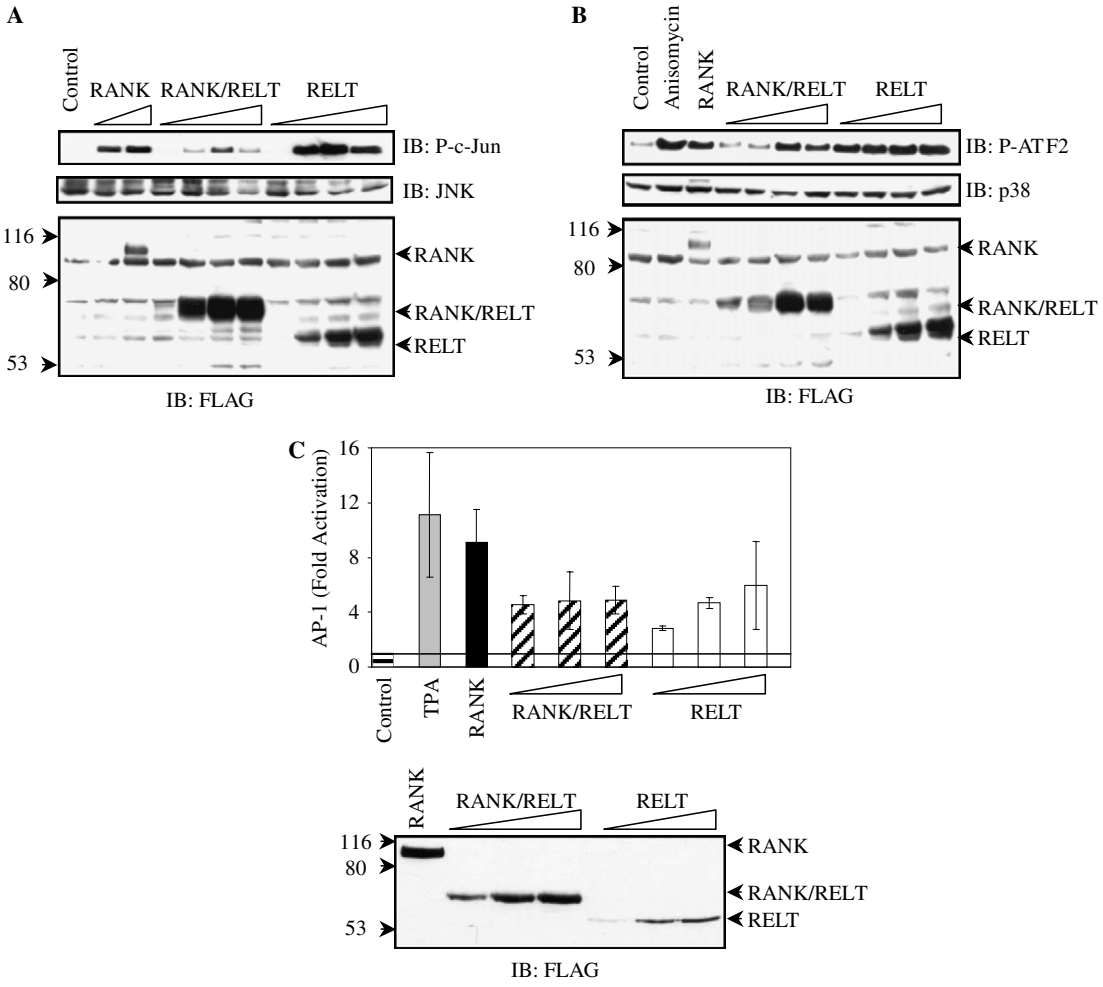

Fig. 1. RELT induces activation of the p38 and JNK MAPK signaling pathways. (A) 293 cells were transfected with 0.5 μg of HA-JNK and increasing amounts (0.1, 0.5, 1.0, and 1.5 μg) of FLAG-RELT or FLAG-RANK/RELT. Cells cotransfected with 0.1 and 0.5 μg of RANK served as positive controls, while cells transfected with HA-JNK only (No Receptor) served as a negative control. Cells were lysed, immunoprecipitated with anti-HA, and subjected to an in vitro kinase assay using GST-Jun as a substrate. Samples were separated by SDS–PAGE, and the signal was detected using anti-phospho-c-Jun antibodies. Lower panel – Western blot (IB) with anti-FLAG antibodies for detection of receptor expression or anti-JNK1 antibodies for kinase levels. (B) As in (A), except that 0.5 μg HA-p38 was transfected into 293 cells, and GST-ATF2 was used as a substrate. 293 cells treated with 1 μg/ml anisomycin for 10 min or transfected with 0.5 μg of RANK, were used as positive controls. Signal was detected using anti-phospho-ATF2. Lower panel – Western blot with anti-FLAG for detection of receptor expression or anti-p38 antibodies for kinase levels. (C) 293 cells were transfected with FLAG-RANK (0.5 μg), FLAG-RANK/RELT (0.5, 1, and 2 μg), or FLAG-RELT (0.5, 1, and 2 μg), together with 0.5 μg of AP-1 luciferase. Cells treated with 250 nM TPA for 24 h served as positive control. Samples were analyzed for luciferase activity using the dual luciferase reporter system (Promega) and activities were normalized to the levels of *Renilla* expression. Lower panel – Anti-FLAG Western blot shows receptor expression.

Sica et al. [4] reported that overexpression of RELT in 293 cells activated the NF-κB pathway as measured using a reporter assay. To confirm those results, we repeated the assays in 293 cells overexpressing RELT or RANK/RELT using both luciferase and secreted alkaline phosphatase (SEAP) reporters. In contrast to the former findings, we could not detect significant NF-κB activation by RELT or RANK/RELT, whereas the positive control, RANK, activated NF-κB very well in the same assays (Figs. 2A and B). Increasing the amount of the RELT or RANK/RELT expression plasmid used for the transfection did not lead to an increase in reporter activity and similar results were seen in HeLa cells (data not shown). Thus, it appears that RELT is a potent activator of the p38 and JNK MAPK pathways, but not NF-κB.

RELT does not bind the TRAF adaptor proteins

TNF receptors lacking DDs generally use TRAFs to transmit signals and activate the NF-κB, p38, and JNK pathways. Therefore, we examined RELT's ability to bind TRAFs 1,2,3,5, and 6 using a GST-pull-down assay. 293 cells were transfected with Myc- or FLAG-tagged TRAF proteins and pulled down with GST-RELT cytoplasmic domain (GST-$RELT_{CD}$) or with GST-$RANK_{CD}$, which served as a positive control [10]. As shown in Fig. 3A, GST-$RELT_{CD}$ did not bind any of the TRAFs, whereas GST-$RANK_{CD}$ was able to bind TRAFs 1,2,3,5, and 6. Additionally, we tested RELT's ability to bind TRAFs in another system in which TRAFs 1 and 2 were translated in vitro with [^{35}S]methionine. Again, no binding of TRAFs

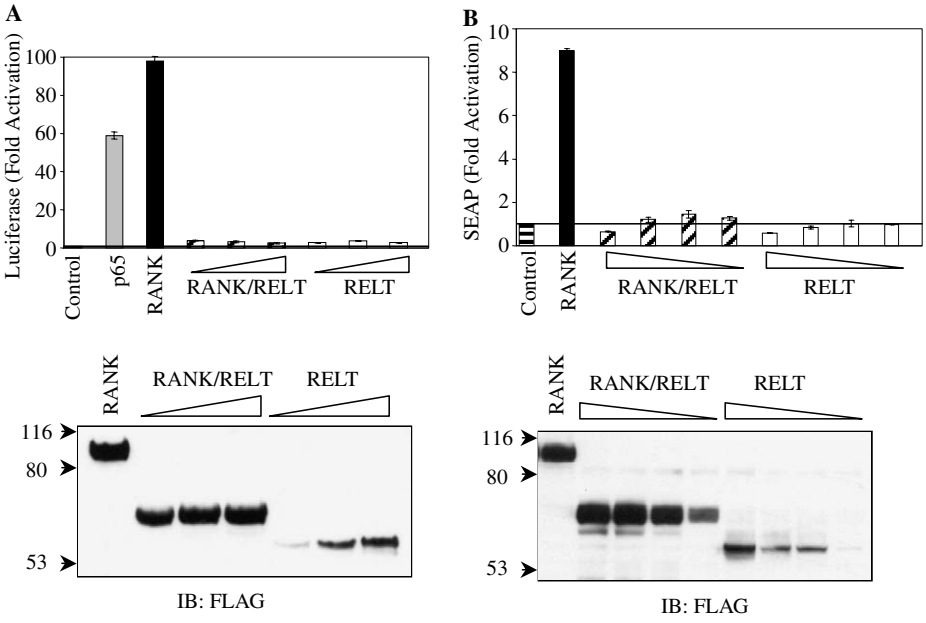

Fig. 2. RELT does not activate the NF-κB pathway. (A) 293 cells were transfected with FLAG-RANK (0.5 μg), FLAG-RANK/RELT (0.5, 1, and 1.5 μg), or FLAG-RELT (0.5, 1, and 1.5 μg), together with 0.5 μg of 3× NF-κB-driven luciferase plasmid. Activity is presented as fold luciferase activation compared with vector-transfected cells. Lower panel – Total cell lysates were separated by SDS–PAGE and probed with anti-FLAG to evaluate receptor expression. (B) As in (A), except that 293 cells were transfected with FLAG-RANK (0.1 μg), FLAG-RANK/RELT (0.1, 0.5, 1, and 2 μg), or FLAG-RELT (0.1, 0.5, 1, and 2 μg), together with 0.5 μg of NF-κB SEAP reporter.

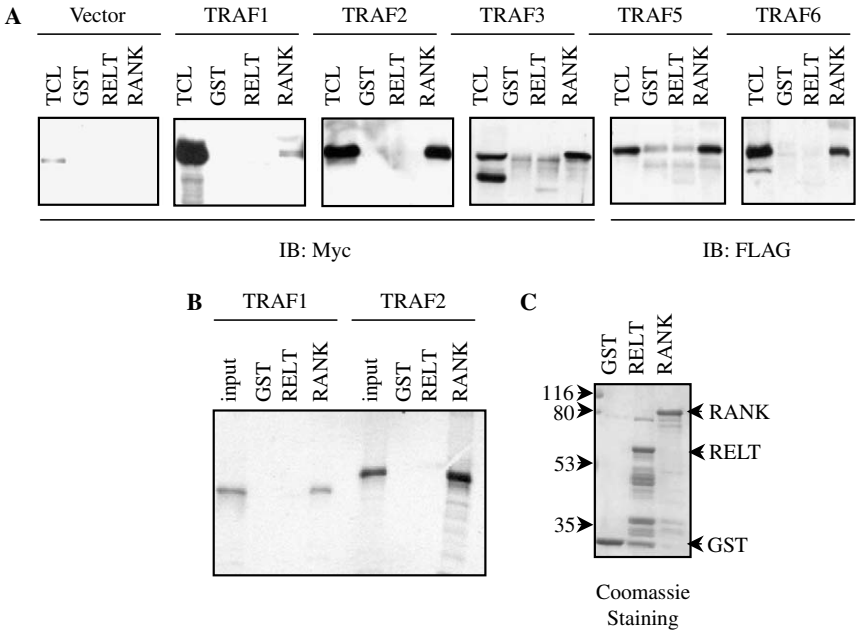

Fig. 3. RELT does not bind the adaptor TRAF molecules. (A) 293 cells were transfected with the vector alone, Myc-tagged hTRAF1, hTRAF2, hTRAF3, or FLAG-tagged mTRAF5 or mTRAF6. GST, GST-$RELT_{CD}$, and GST-$RANK_{CD}$ were used to pull down the TRAFs. Total cell lysates (TCL) show TRAF expression levels. The samples were analyzed by SDS–PAGE and Western blotting with either anti-Myc or anti-FLAG antibodies. (B) hTRAF1 and hTRAF2 were translated in vitro and labeled with [^{35}S]methionine. Labeled TRAFs were pulled down with GST-$RELT_{CD}$ or GST-$RANK_{CD}$ and samples were analyzed by SDS–PAGE and autoradiography. Ten percent of the total input was run to show TRAF expression levels. (C) Coomassie brilliant blue staining of the equivalent amounts of GST, GST-$RELT_{CD}$, and GST-$RANK_{CD}$ that were used in the assay is shown.

to GST-$RELT_{CD}$ was detected, whereas GST-$RANK_{CD}$ pulled down [^{35}S]TRAFs 1 and 2 (Fig. 3B). This lack of binding of RELT to TRAFs was not due to low expression, since all the TRAFs were highly expressed and were able to bind RANK.

RELT binds to SPAK and is phosphorylated by SPAK

To identify new molecules that bind RELT and may be involved in RELT signaling, we used the Ras recruitment system (RRS) [9]. The RRS is a yeast two-hybrid system

that is based on the strict requirement that RAS be localized to the plasma membrane for its function, and is suitable for studying interactions between transmembrane receptors and cytoplasmic proteins. We screened 1×10^5 transformants and found 50 clones that grew on galactose in the absence of methionine at 37 °C when cotransformed with $RELT_{CD}$, but not vector alone. These clones were sequenced, and pertinent candidates were translated in vitro with [^{35}S]methionine and screened for interaction using GST-$RELT_{CD}$. Of these clones, one that codes for the C-terminal domain (amino acids 322–547) of SPAK consistently interacted with the GST-$RELT_{CD}$ (Figs. 4A and B). This interaction was specific since SPAK bound to RELT, but not to RANK (Fig. 4B).

To further verify the interaction between SPAK and RELT, Myc-SPAK was transfected into 293 cells stably expressing FLAG-RELT or RANK/RELT. Immunoprecipitations were performed with anti-FLAG antibodies, followed by anti-Myc Western blotting. As shown in Fig. 4C, both RELT and RANK/RELT were able to bind SPAK. In addition, OSR1, the closest kinase relative of SPAK, was found to bind weakly to RANK/RELT. No binding of OSR1 to RELT was detected, probably because of the lower level of RELT expression as compared with RANK/RELT.

RELT has the sequence ^{349}RFRV in its intracellular domain which is similar to the consensus motif (R/K)FX(V/I) shown previously to mediate the binding of SPAK to members of the cation chloride cotransporters superfamily [11]. A single amino acid mutation (phenylalanine to alanine) within this sequence eliminated the binding of SPAK to two Na–K–2Cl cotransporters (NKCC1 and 2) and to a K-Cl cotransporter (KCC3). To determine whether the binding of SPAK to RELT is mediated by the ^{349}RFRV motif, we generated the mutant RELT(F/A). Similar to the binding of SPAK to ion cotransporters, the F350A

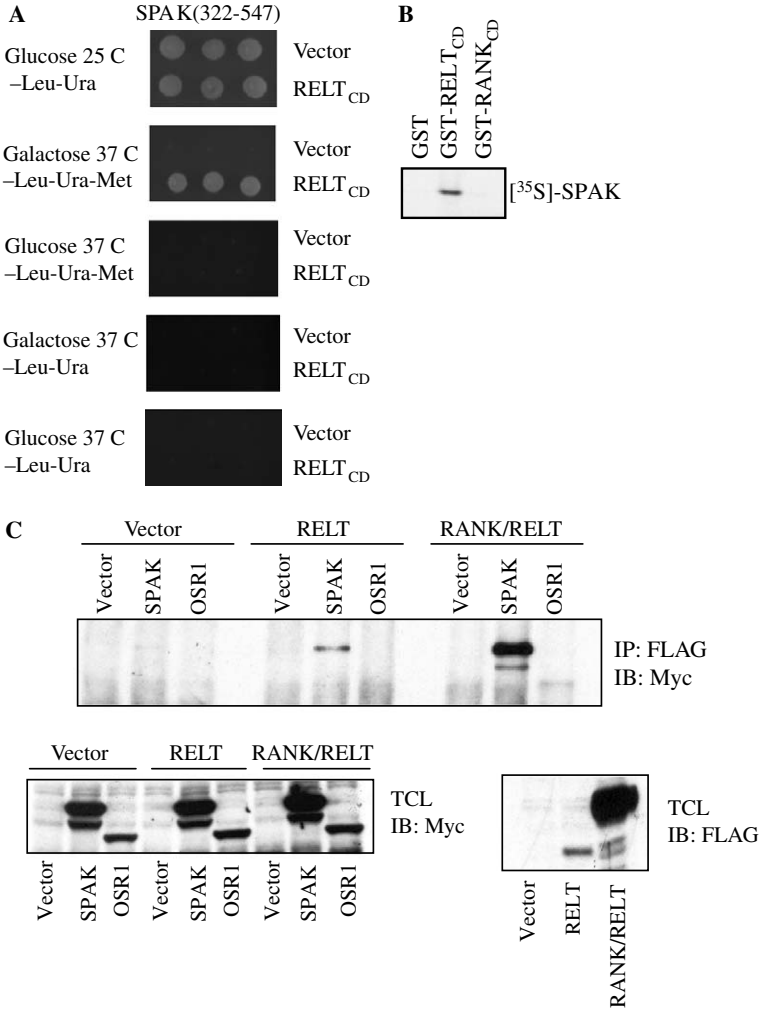

Fig. 4. RELT and RANK/RELT interact specifically with SPAK. (A) Cotransformation of the cdc25H yeast strain with the isolated library clone pMyr-SPAK(322–547), together with either the vector or Myc-Ras-$RELT_{CD}$. The yeasts were then analyzed for growth in triplicate as described under Experimental procedures. (B) pMyr-SPAK(322–547), translated in vitro and labeled with [^{35}S]methionine, was pulled down with GST, GST-$RELT_{CD}$, or GST-$RANK_{CD}$, and binding was detected using autoradiography. (C) 293 cells stably expressing the vector alone, FLAG-RELT, or FLAG-RANK/RELT were transfected with Myc-tagged mSPAK or hOSR1. Anti-FLAG was used in a co-immunoprecipitation assay, and binding of RELT to SPAK or OSR1 was determined using anti-Myc. Total cell lysates were probed with anti-Myc and anti-FLAG antibodies to verify protein expression.

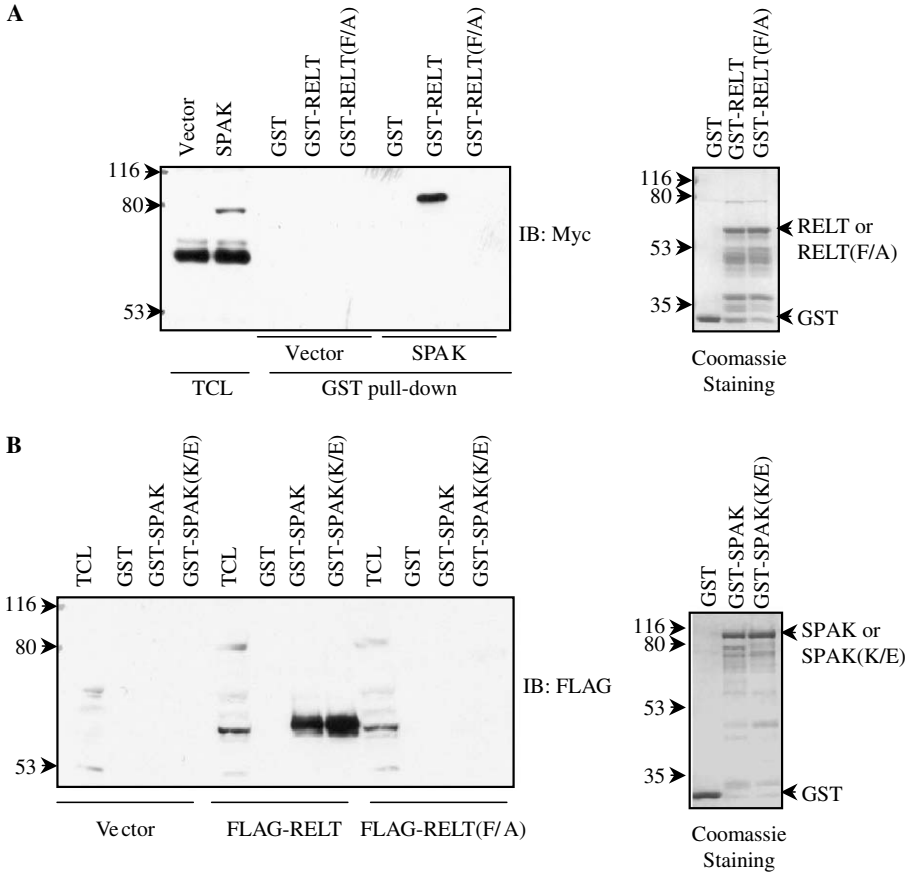

Fig. 5. A single point mutation in RELT, F350A, eliminates binding to SPAK. (A) 293 cells transfected with Myc-SPAK or the vector alone were lysed and incubated with GST, GST-RELT$_{CD}$, or GST-RELT(F/A)$_{CD}$. Binding was detected using anti-Myc Western blotting. Coomassie brilliant blue staining of the equivalent amounts of GST, GST-RELT and GST-RELT(F/A) that were used in the assay. (B) 293 cells transfected with FLAG-RELT or FLAG-RELT(F/A) were lysed and incubated with GST, GST-SPAK, or GST-SPAK(K/E). Binding was detected using anti-FLAG Western blotting. Coomassie brilliant blue staining of the equivalent amounts of GST, GST-SPAK, and GST-SPAK(K/E) is shown.

mutation in RELT completely eliminated the binding of Myc-SPAK to GST-RELT(F/A)$_{CD}$ (Fig. 5A). Moreover, in the complementary experiment, GST-SPAK bound to FLAG-RELT but not FLAG-RELT(F/A) (Fig. 5B). Additionally, the kinase-dead SPAK, GST-SPAK(K/E) also bound FLAG-RELT, but not FLAG-RELT(F/A) (Fig. 5B).

SPAK interacts with and is a substrate of the "with no K (lysine) protein kinases," WNK1 and WNK4, which are mutated in Gordon's hypertension syndrome [12,13]. In a recent study, WNK1 and WNK4 were shown to phosphorylate SPAK and OSR1 on two residues: ^{233}T and ^{373}S on SPAK and ^{185}T and ^{325}S on OSR1 [7]. Phosphorylation on residue ^{185}T of OSR1, which is located in the activation loop of the kinase catalytic domain, mediated OSR1 activation. To test whether phosphorylation at the activation loop is also required for SPAK's catalytic activity, we constructed a SPAK mutant, GST-SPAK(T/E), in which residue ^{243}T (the mouse residue corresponding to human ^{233}T) was mutated to glutamic acid. As seen in Fig. 6A, the activity of GST-SPAK(T/E) was much higher than the activity of GST-SPAK as judged by both autophosphorylation and phosphorylation of the SPAK substrate NKCC1, suggesting that phosphorylation of residue ^{243}T increases the catalytic activity of SPAK.

Next, we performed an in vitro kinase assay to find out whether RELT is a substrate for SPAK. The abilities of GST, GST-SPAK, GST-SPAK(K/E), and GST-SPAK(T/E) to phosphorylate GST, GST-RELT$_{CD}$, and NKCC1 were tested. As expected, GST and kinase dead GST-SPAK(K/E), did not phosphorylate any of the exogenous substrates (Figs. 6A and B). GST-SPAK exhibited a weak autophosphorylation activity, but did not phosphorylate GST, GST-RELT$_{CD}$ or NKCC1. However, the constitutively active GST-SPAK(T/E) phosphorylated both GST-RELT$_{CD}$ and NKCC1, but not GST, suggesting that the RELT$_{CD}$ may be a substrate for SPAK.

SPAK is involved in RELT-induced p38 and JNK activation

To determine the role of SPAK in RELT-activated signaling pathways, we first investigated the ability of the RELT(F/A) mutant, which cannot bind SPAK, to activate JNK and p38 signaling. As shown in Fig. 7A, impaired activation of the p38 and JNK kinase pathways was observed in 293 cells overexpressing RANK/RELT(F/A),

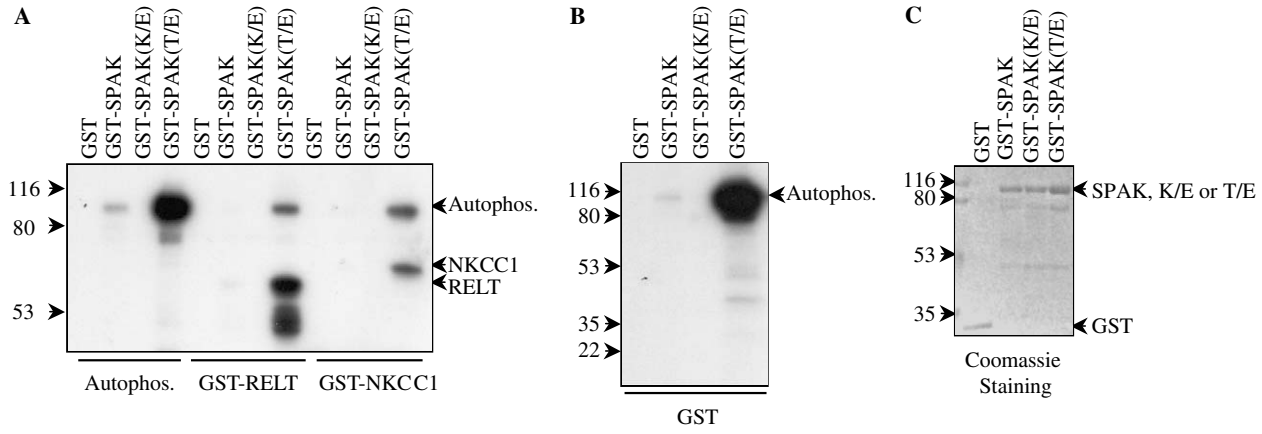

Fig. 6. RELT is phosphorylated by SPAK. An in vitro kinase assay was performed using GST, GST-SPAK, kinase inactive GST-SPAK(K/E), and constitutively active GST-SPAK(T/E). (A) Proteins were incubated with either no substrate (to detect autophosphorylation), GST-RELT or GST-NKCC1 and analyzed by 8% SDS–PAGE and autoradiography. (B) Proteins were incubated with GST as a negative control for substrate phosphorylation and analyzed by 12% SDS–PAGE and autoradiography. (C) Coomassie brilliant blue staining of the equivalent amounts of GST, GST-SPAK, GST-SPAK(K/E), and GST-SPAK(T/E) is shown.

but not in cells expressing RANK/RELT. To further investigate the connection between RELT signaling and SPAK, we examined the effect of overexpression of the SPAK kinase-dead mutant, SPAK(K/E), on RELT signaling in 293 cells (Fig. 7B). Increased expression of SPAK(K/E) inhibited RELT-induced activation of the p38 and JNK signaling cascades in a dose-dependent manner, thereby acting in a dominant-negative fashion. In contrast, SPAK(K/E) expression had little effect on the signaling induced by RANK, suggesting that this inhibition is specific for RELT and is not a general effect on signaling by other TNFRs.

Discussion

TNFRs are devoid of intrinsic kinase activity and typically engage TRAFs to regulate downstream kinase signaling cascades. In this study, we present the first example of a TNFR family member, RELT, which uses a kinase, SPAK, instead of TRAFs to activate p38 and JNK signaling. Interestingly, inspection of the intracellular domains of all TNFRs revealed that RELT is the only receptor containing the (R/K)FX(V/I) SPAK-binding consensus site, suggesting that RELT is the only TNFR that binds SPAK.

TNFRs lacking DDs usually activate both the MAPK and NF-κB signaling pathways and bind to TRAFs [1,14]. We showed, however, that RELT, despite lacking a DD, is unable to activate the NF-κB pathway (Fig. 2) or bind TRAFs (Fig. 3). These results contradict those published by Sica et al. [4], who showed that overexpression of RELT in 293 cells activates NF-κB and that TRAF1 (but not TRAF2,3,5, or 6) binds RELT. We confirmed the lack of NF-κB activation by RELT using two different reporter assays, luciferase and SEAP, in both 293 and HeLa cells (Fig. 2 and data not shown). In addition, a wide range of RELT and RANK/RELT protein expression levels were tested, as was stimulation of cells expressing the RANK/RELT chimera with RANK ligand (data not shown). Nevertheless, although overexpression of RANK or the p65 subunit of NF-κB induced considerable NF-κB activation, we were unable to detect significant NF-κB activation by RELT or RANK/RELT (Fig. 2). We cannot exclude the possibility that in other cell types RELT is able to activate NF-κB, yet, under conditions in which other TNFRs lacking DDs readily induce NF-κB activation (e.g., overexpression in 293 cells), RELT is unable to do so.

NF-κB activation by TNFRs is primarily mediated by the recruitment of TRAFs [15–17]. Therefore, the inability of RELT to activate NF-κB is consistent with the observation that RELT does not bind TRAFs. We tested the interactions between TRAFs and RELT using a GST-$RELT_{CD}$ pull-down assay with TRAFs 1,2,3,5, or 6 (Fig. 3A) and in an in vitro transcription/translation assay with TRAFs 1 and 2 (Fig. 3B). Still, we were consistently unable to detect any TRAF binding to RELT, although RANK did bind to all the TRAFs under these conditions. In addition, our results (except for TRAF1) also agreed with those of Sica et al., who could not detect binding of RELT to TRAFs 2,3,5, and 6.

SPAK, a serine/threonine kinase in the Ste20 family, was identified in a yeast two-hybrid screen as a protein that binds RELT's cytoplasmic domain (Fig. 4A). SPAK is a 60-kDa kinase with an N-terminal proline/alanine-rich region, followed by a kinase domain, a putative nuclear localization signal, and a potential caspase cleavage site (^{399}DEMDE) [18]. SPAK binding to RELT was verified using GST-pull-down and coimmunoprecipitation assays (Fig. 4). The interaction was mapped to ^{349}RFAV in RELT's cytoplasmic domain which is similar to the consensus motif (R/K)FX(V/I) found in members of the cation chloride cotransporters [11] (Fig. 5). In addition, binding of RELT to SPAK was mapped to the last 154 C-terminal amino acids in mouse SPAK based on the cloned human SPAK library plasmid (amino acids 322–547) identified

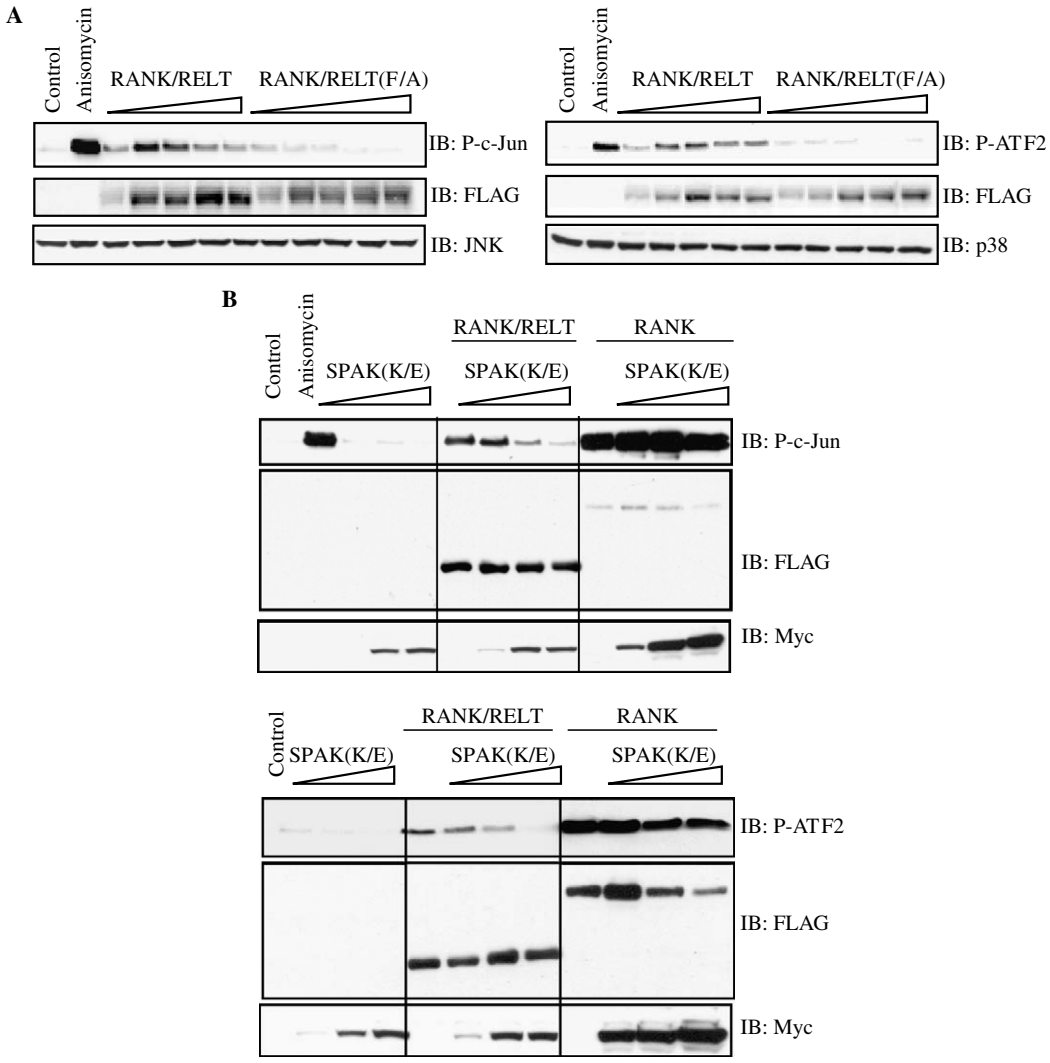

Fig. 7. SPAK is involved in RELT signaling. (A) 293 cells were transfected with FLAG-RANK/RELT (0.125, 0.25, 0.5, 0.75, and 1 μg) or FLAG-RANK/RELT(F/A) (0.25, 0.5, 0.75, 1, and 1.25 μg) and either 0.5 μg of HA-JNK or HA-p38 as indicated. Cell lysates were immunoprecipitated with anti-HA, and an in vitro kinase assay was performed using GST-c-Jun or GST-ATF2 as substrates. Phosphorylation was detected by Western blotting with anti-phospho-c-Jun or anti-phospho-ATF2. 293 cells treated with anisomycin for 10 min were used as positive controls. Receptor expression was evaluated by Western blotting with anti-FLAG antibodies and kinase levels were evaluated with anti-JNK1 or anti-p38 antibodies. (B) 293 cells were transfected with FLAG-RANK/RELT, FLAG-RANK, and increasing amounts of Myc-SPAK(K/E) (0.1, 0.5, and 1 μg), together with 0.5 μg of HA-JNK or HA-p38. In vitro kinase assays were performed as in (A). Expression of the receptors was examined by Western blotting with anti-FLAG antibodies, and SPAK(K/E) expression was determined using anti-Myc antibodies.

by the yeast screen and on the observation that a SPAK mutant, SPAKΔC_{402}, ending at the potential caspase cleavage site did not bind RELT (data not shown). This C-terminal sequence in SPAK contains no known motifs, and further studies are required to narrow down the interacting region and determine the role that caspase cleavage plays in SPAK regulation.

SPAK is a member of the Ste20 family of kinases that includes germinal center kinases (GCKs) and p21-activated kinases (PAKs) [19]. Ste20 was originally identified in yeast as a mitogen-activated protein kinase kinase kinase kinase (MAP4K) [20] and several other mammalian homologues of Ste20 have also been shown to be upstream regulators of MAPK cascades, including hematopoietic progenitor kinase-1 (HPK1), germinal center kinase-like kinase, and PAK2 [19].

Little is known about the function and regulation of SPAK. However, like other members of the Ste20 group of kinases, SPAK has been shown to activate the p38 and JNK signaling cascades [6,11,12,21,22]. In T cells, SPAK has been shown to associate constitutively with PKCθ and to serve as a substrate of PKCθ in T-cell receptor-induced AP-1 activation [6]. In addition, SPAK regulates the activity of ion transporters such as NKCC1 and KCC2 through its interaction with WNKs [13,23].

Our results suggest a function for SPAK in mediating RELT-induced activation of the p38 and JNK signaling cascades. Overexpression of RELT(F/A), which is

incapable of binding SPAK, greatly reduced RELT's ability to activate the p38 and JNK signaling pathways (Fig. 7A). Furthermore, a kinase-dead mutant of SPAK inhibited p38 and JNK signaling by RELT, but not RANK (Fig. 7B). Ste20 family members have been shown to play important roles downstream of TNFRs, although SPAK is the only member so far that has been shown to bind a TNFR directly [24–28].

The molecular mechanism of the signaling induced by RELT and SPAK is as yet unclear. We found that the RELT is phosphorylated by SPAK in an in vitro kinase assay (Fig. 6); however, the effect of phosphorylation on RELT's activity is still not known. SPAK appears to be constitutively bound to RELT and may be activated by binding of a putative RELT ligand. Alternatively, WNK1, WNK4, or some other kinase may be involved in regulating SPAK activity by phosphorylation. The constitutively active SPAK mutant, SPAK(T/E), supports the notion that phosphorylation at the activation loop is required for SPAK's catalytic activity (Fig. 6). In addition, the activity of SPAK may be regulated by caspase cleavage of a C-terminal regulatory domain in a manner similar to other members of the Ste20 family, such as PAK2, HPK-1, Mst, and SLK [25–32]. It is possible that both proteolysis and phosphorylation are required for full activation.

Classification of TNFRs is based on the presence of an intracellular DD that serves to distinguish between two classes of receptors: those with DDs and those lacking DDs, also called TRAF-binding receptors. Because DDs are associated with caspase activation and TRAFs with NF-κB, p38, and JNK activation, this classification involves structural features and has implications for receptor function [16,17,33,34]. Using this classification scheme, it was predicted that a non-DD-containing TNFR such as RELT would bind TRAFs and activate the NF-κB, p38, and JNK pathways. However, our results suggest that RELT does not depend on TRAFs for signaling, but uses SPAK for p38 and JNK activation. We believe that RELT may represent a new group of TNFRs that has further diverged from the standard signaling scheme typical of other TNFRs. Future studies will entail analysis of the molecular mechanism by which RELT transmits signals for activation of SPAK and downstream signaling pathways.

Acknowledgment

We thank Gayle Nesom from The University of Texas M.D. Anderson Cancer Center Department of Scientific Publications for excellent editorial assistance.

References

[1] B.B. Aggarwal, Signalling pathways of the TNF superfamily: a double-edged sword, Nat. Rev. Immunol. 3 (2003) 745–756.
[2] R.M. Locksley, N. Killeen, M.J. Lenardo, The TNF and TNF receptor superfamilies: integrating mammalian biology, Cell 104 (2001) 487–501.
[3] J.L. Bodmer, P. Schneider, J. Tschopp, The molecular architecture of the TNF superfamily, Trends Biochem. Sci. 27 (2002) 19–26.
[4] G.L. Sica, G. Zhu, K. Tamada, D. Liu, J. Ni, L. Chen, RELT, a new member of the tumor necrosis factor receptor superfamily, is selectively expressed in hematopoietic tissues and activates transcription factor NF-kappa B, Blood 97 (2001) 2702–2707.
[5] B.G. Darnay, J. Ni, P.A. Moore, B.B. Aggarwal, Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif, J. Biol. Chem. 274 (1999) 7724–7731.
[6] Y. Li, J. Hu, R. Vita, B. Sun, H. Tabata, A. Altman, SPAK kinase is a substrate and target of PKCtheta in T-cell receptor-induced AP-1 activation pathway, EMBO J. 23 (2004) 1112–1122.
[7] A.C. Vitari, M. Deak, N.A. Morrice, D.R. Alessi, The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome, phosphorylate and activate SPAK and OSR1 protein kinases, Biochem. J. (2005).
[8] T.C. Polek, M. Talpaz, B.G. Darnay, T. Spivak-Kroizman, TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TweakR. Evidence for a second TWEAK receptor, J. Biol. Chem. 278 (2003) 32317–32323.
[9] Y.C. Broder, S. Katz, A. Aronheim, The ras recruitment system, a novel approach to the study of protein–protein interactions, Curr. Biol. 8 (1998) 1121–1124.
[10] B.G. Darnay, B.B. Aggarwal, Signal transduction by tumour necrosis factor and tumour necrosis factor related ligands and their receptors, Ann. Rheum. Dis. 58 (Suppl. 1) (1999) I2–I13.
[11] K. Piechotta, J. Lu, E. Delpire, Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1), J. Biol. Chem. 277 (2002) 50812–50819.
[12] K. Piechotta, N. Garbarini, R. England, E. Delpire, Characterization of the interaction of the stress kinase SPAK with the Na^+–K^+–2Cl- cotransporter in the nervous system: evidence for a scaffolding role of the kinase, J. Biol. Chem. 278 (2003) 52848–52856.
[13] K.B. Gagnon, R. England, E. Delpire, Volume sensitivity of cation-chloride cotransporters is modulated by the interaction of two kinases: SPAK and WNK4, Am. J. Physiol. Cell Physiol. (2005).
[14] U. Gaur, B.B. Aggarwal, Regulation of proliferation, survival and apoptosis by members of the TNF superfamily, Biochem. Pharmacol. 66 (2003) 1403–1408.
[15] T. Hehlgans, K. Pfeffer, The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games, Immunology 115 (2005) 1–20.
[16] J.Y. Chung, Y.C. Park, H. Ye, H. Wu, All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction, J. Cell Sci. 115 (2002) 679–688.
[17] J.R. Bradley, J.S. Pober, Tumor necrosis factor receptor-associated factors (TRAFs), Oncogene 20 (2001) 6482–6491.
[18] H. Ushiro, T. Tsutsumi, K. Suzuki, T. Kayahara, K. Nakano, Molecular cloning and characterization of a novel Ste20-related protein kinase enriched in neurons and transporting epithelia, Arch. Biochem. Biophys. 355 (1998) 233–240.
[19] I. Dan, N.M. Watanabe, A. Kusumi, The Ste20 group kinases as regulators of MAP kinase cascades, Trends Cell Biol. 11 (2001) 220–230.
[20] C. Wu, M. Whiteway, D.Y. Thomas, E. Leberer, Molecular characterization of Ste20p, a potential mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK) kinase kinase from *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 15984–15992.
[21] A.M. Johnston, G. Naselli, L.J. Gonez, R.M. Martin, L.C. Harrison, H.J. DeAizpurua, SPAK, a STE20/SPS1-related kinase that activates the p38 pathway, Oncogene 19 (2000) 4290–4297.
[22] W. Chen, M. Yazicioglu, M.H. Cobb, Characterization of OSR1, a member of the mammalian Ste20p/germinal center kinase subfamily, J. Biol. Chem. 279 (2004) 11129–11136.

[23] B.F. Dowd, B. Forbush, PASK (proline-alanine-rich STE20-related kinase), a regulatory kinase of the Na–K–Cl cotransporter (NKCC1), J. Biol. Chem. 278 (2003) 27347–27353.
[24] T. Yuasa, S. Ohno, J.H. Kehrl, J.M. Kyriakis, Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38, J. Biol. Chem. 273 (1998) 22681–22692.
[25] J.D. Graves, Y. Gotoh, K.E. Draves, D. Ambrose, D.K. Han, M. Wright, J. Chernoff, E.A. Clark, E.G. Krebs, Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1, EMBO J. 17 (1998) 2224–2234.
[26] K.K. Lee, M. Murakawa, E. Nishida, S. Tsubuki, S. Kawashima, K. Sakamaki, S. Yonehara, Proteolytic activation of MST/Krs, STE20-related protein kinase, by caspase during apoptosis, Oncogene 16 (1998) 3029–3037.
[27] Y.R. Chen, C.F. Meyer, B. Ahmed, Z. Yao, T.H. Tan, Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1), Oncogene 18 (1999) 7370–7377.
[28] L.A. Sabourin, K. Tamai, P. Seale, J. Wagner, M.A. Rudnicki, Caspase 3 cleavage of the Ste20-related kinase SLK releases and activates an apoptosis-inducing kinase domain and an actin-disassembling region, Mol. Cell. Biol. 20 (2000) 684–696.
[29] T. Rudel, G.M. Bokoch, Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2, Science 276 (1997) 1571–1574.
[30] L.A. Sabourin, M.A. Rudnicki, Induction of apoptosis by SLK, a Ste20-related kinase, Oncogene 18 (1999) 7566–7575.
[31] K.K. Lee, T. Ohyama, N. Yajima, S. Tsubuki, S. Yonehara, MST, a physiological caspase substrate, highly sensitizes apoptosis both upstream and downstream of caspase activation, J. Biol. Chem. 276 (2001) 19276–19285.
[32] P.M. de Souza, M.A. Lindsay, Mammalian Sterile20-like kinase 1 and the regulation of apoptosis, Biochem. Soc. Trans. 32 (2004) 485–488.
[33] J.R. Muppidi, J. Tschopp, R.M. Siegel, Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction, Immunity 21 (2004) 461–465.
[34] A. Bhardwaj, B.B. Aggarwal, Receptor-mediated choreography of life and death, J. Clin. Immunol. 23 (2003) 317–332.