Mol. Cells, Vol. 14, No. 3, pp. 420-424

Communication

Molecules and Cells

RACK-1, a Receptor for Activated C Kinase, Interacts with the Transcription Factor NFAT and Represses Its Transactivation

Juhyun Han, Minjung Kim, Ji-Hyun Oum, Heejoon Myung, Seong-Wook Lee[1], Sunjoo Jeong[1], Surendra Sharma[2], and Jungchan Park*

Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yongin 449-791, Korea;
[1] Department of Molecular Biology, Dankook University, Seoul 140-714, Korea;
[2] Departments of Pediatrics and Pathology, Brown University, Women and Infants' Hospital of Rhode Island, Providence, RI 02905, USA.

(Received March 6, 2002; Accepted September 2002)

To isolate and characterize a novel protein that interacts with nuclear factor of activated T cells (NFAT) and potentially regulates its activity, we screened a Jurkat cDNA library by using the NFAT regulatory domain as bait in the yeast two-hybrid system. RACK-1, a receptor for activated protein kinase C and a homologue of the G-protein β subunit, was identified as a NFAT-binding protein. Mammalian two hybrid tests in CV-1 cells and a coimmunoprecipitation assay confirmed protein-protein interaction between NFAT and RACK-1. In addition, overexpression of RACK-1 specifically suppressed transcriptional activation derived by NFAT, but not by NF-κB. These results demonstrate RACK-1 as a potent negative modulator of NFAT activation and suggest a novel mechanism in NFAT regulation.

Keywords: NFAT; RACK-1; Transcription Factor; Yeast-Two Hybrid.

Introduction

The nuclear factor of activated T cells (NFAT) family of transcription factors is required for the expression of numerous cytokine genes and cell surface molecules that play a pivotal role in both the initiation and coordination of the immune response (Crabtree, 1999; Rao *et al*., 1997). NFAT proteins are regulated primarily at the levels of their subcellular localization and DNA binding that are sensitive to their phosphorylation states (Park *et al*., 1995; Shibasaki *et al*., 1996; Timmerman, *et al*., 1996; Zhu and McKeon, 1999). In resting T cells, NFAT is phosphorylated at multiple serine residues and localized in the cytoplasm. Antigenic stimulus that causes T cell activation and the accompanying increase of intracellular calcium lead to dephosphorylation of NFAT by Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin and subsequent translocation of NFAT into the nucleus. However, sustained calcineurin activity is necessary to maintain NFAT in an active state. When calcineurin loses its phosphatase activity by removing the stimulus or treating cells with the immunosuppressive drugs cyclosporin A and FK-506 that inactivate calcineurin, NFAT is rapidly rephosphorylated and exported from the nucleus (Loh *et al*., 1996; Shibasaki *et al*., 1996; Timmerman *et al*., 1996). The major sites of dynamic phosphorylation have been localized at the N-terminal regulatory domain that is highly conserved in the classical NFAT family members (NFATp, NFATc, NFAT3, and NFAT4/NFATx) (Okamura *et al*., 2000). Recently, although several candidates of NFAT kinases such as GSK-3, CK1α, and JNK2 have been identified to promote phosphorylation and export of NFAT from the nucleus (Beals *et al*., 1997; Chow *et al*., 1997; Zhu *et al*., 1998), it is unclear whether these protein kinases are able to phosphorylate all the NFAT family members.

Moreover, although dynamic changes in phosphorylation status of NFAT play a pivotal role in its regulation, several lines of evidence demonstrate that NFAT activity is also modulated by other additional proteins including

* To whom correspondence should be addressed.
Tel: 82-31-330-4355; Fax: 82-31-333-1696
E-mail: jpark@hufs.ac.kr

Abbreviations: CK, casein kinase; DBD, DNA binding domain; GSK, glycogen synthase kinase; JNK, Jun N-terminal kinase; NFAT, nuclear factor of activated T cells; PMA, phorbol 12-myristate 13-acetate; RACK, receptor for activated C kinase.

nuclear coactivator p300, GATA proteins, and nuclear export receptor Crm1 (Garcia-Rodriguez and Rao, 1998; Musaro *et al*., 1999; Zhu and McKeon, 1999), thus suggesting that transcriptional activity of NFAT is regulated at multiple steps. Using N-terminal regulatory domain of NFATc as bait in yeast two-hybrid system, we identified here RACK-1 as a novel protein that interacts with NFAT. Association of RACK-1 with NFAT was demonstrated by coimmunoprecipitation and two-hybrid tests in mammalian cells. Overexpression of RACK-1 in CV-1 cells selectively repressed transcriptional activity of NFAT in response to stimulation with PMA and ionomycin, suggesting a novel function of RACK-1 in NFAT regulation.

Materials and Methods

Cells culture and transfection Monkey kidney fibroblast CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. CV-1 cells (5×10^4 cells/well) were seeded in a 24-well plate. After incubation for 16–18 h, exponentially growing cells were transfected with 0.25 μg of each of the plasmids, pNFAT-Luc, NFATc.β expression vector (Park *et al*., 1996), pCH110 (Pharmacia, USA), and either pcDNA3 (Invitrogen, USA) or Myc-tagged RACK expression vector, by the DMRIE-C reagent (Gibco-BRL, USA) as described in the manufacturer's protocol. To assess expression of NFATc.β and myc-RACK-1 in CV-1 cells after transfection, immunoblot assays were performed with anti-NFAT antibody (Santa Cruz, USA) or anti-myc monoclonal antibody (Invitrogen, USA). The reporter plasmid pNFAT-Luc expresses luciferase driven by three copies of IL-2 NFAT sites upstream of thymidine kinase minimal promoter, and the p3X-kB-Luc is under control of a minimal *fos* promoter with three copies of the major histocompatability complex class I kB element (Mitchell and Sugden, 1995). To normalize transfection efficiency, pCH110 was used. At 24 h after transfection, cells were stimulated with 25 ng/ml of PMA (Sigma, USA) and 1 μM of ionomycin (Calbiochem, USA) for 7 h. Cells were lysed in reporter lysis buffer, and enzyme activity of luciferase and β-galactosidase was determined with a luciferase assay kit (Promega, USA) and with 4-methyl-lumbellifery-β-galactoside (MUG) as a substrate, respectively (Oum *et al.*, 2002).

Mammalian two-hybrid assays To generate pG4-NFAT expressing a fusion protein of NFATc.β and GAL4 DNA binding domain as bait, PCR-amplified DNA fragment encoding NFATc.β (amino acids 104–812) was cloned into pGAL4 DBD (Luo *et al*., 1996) between *Bam*HI and *Xba*I sites. For a vector pVP16-RACK expressing target protein, PCR product encoding RACK-1 (amino acids 68–293) was inserted into pVP16 (Clontech, USA), which expresses a fusion protein of RACK-1 and the VP16 trans-activating domain. CV-1 cells were transfected with 0.5 μg of each of the plasmids, pG4-NFAT, pVP16-RACK, a reporter pGal4-*tk*-Luc, and pCH110 for normalizing transfection efficiency. At 24 h after transfection, luciferase activity was determined.

Immunoprecipitation and immunoblot Plasmids expressing myc-tagged full-length RACK-1 and HA-tagged NFATc.β (1–395) were generated in the vector frame of pcDNA3, and myc-RACK-1 and HA-NFATc.β proteins were produced individually by using *in vitro* TNT Quick coupled transcription/translation system (Promega, USA). The same volume (20 μl) of each reaction mixture was mixed in various combination as indicated in Fig. 2, and the mixtures were first immunoprecipitated with anti-myc monoclonal antibody (Invitrogen, USA) and protein G (Sigma, USA). The immunoprecipitation products were disrupted by boiling with 1% SDS lysis buffer for 5 min, and separated in 12% SDS-PAGE. HA-tagged NFAT proteins were detected by an immunoblot assay with rabbit anti-HA antibody (Santa Cruz), and visualized by ECL detection system (Park *et al.*, 1995).

Results and Discussion

Interaction between NFAT and RACK-1 In an attempt to identify a novel protein involved in regulation of NFAT activation, a Jurkat T cell cDNA library was screened with the N-terminal regulatory domain of NFATc as bait in the yeast-two hybrid system. Among a number of positive yeast clones, two different clones encoded the C-terminal 226 amino acids of RACK-1, originally described as a receptor for activated C kinase (Mochly-Rosen *et al.*, 1991; Ron *et al*., 1994). To confirm the interaction between NFAT and RACK-1 in mammalian cells, we performed mammalian two-hybrid tests (Fig. 1A). Cotransfection of pVP16-RACK and pG4-NFAT, which express fusion proteins of VP16 transactivation domain and RACK-1 and of Gal4 DNA binding domain and NFATc.β, respectively, into CV-1 cells led to induction of the GAL4-tk-Luc reporter gene activity. Neither control transfection of pVP16 plus pG4-NFAT nor transfection with single expression vector significantly induced the luciferase activity.

In addition, we also carried out coimmunoprecipitation experiments (Fig. 1B). Using an *in vitro* transcription/translation system from reticulocyte lysate, Myc-tagged RACK and HA-tagged NFAT were produced and immunoprecipitated with anti-myc monoclonal antibodies. An immunoblot analysis of the precipitates with rabbit anti-HA antibodies revealed that approximately 50% of NFAT proteins added to the reaction were coimmunoprecipitated with RACK-1 when compared with protein amounts of the input. The interaction between NFAT and RACK-1 was specific because neither preimmune mouse IgG nor anti-myc antibody itself precipitated HA-tagged NFAT proteins. Taken together with the data of two-hybrid

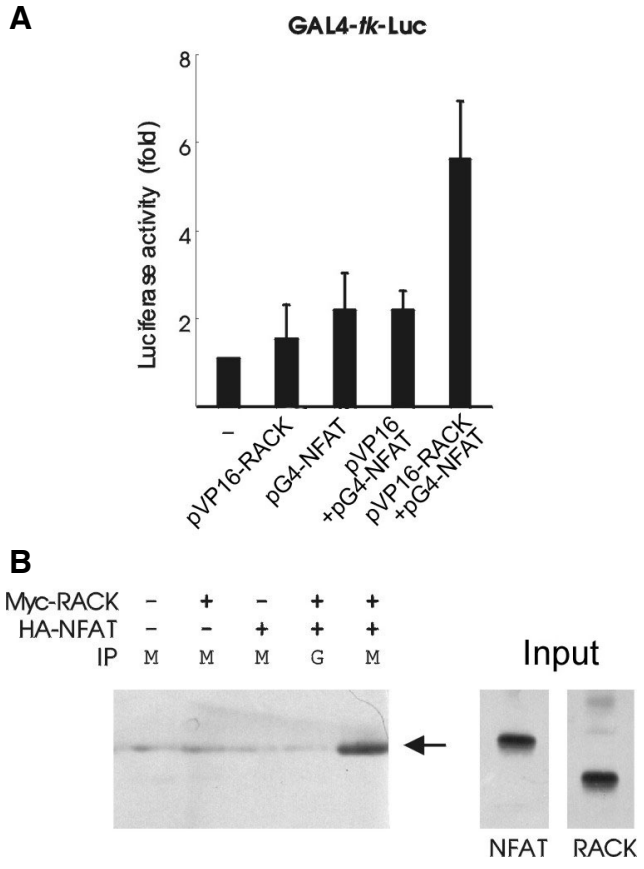

Fig. 1. RACK-1 associates with NFAT. **A.** Two-hybrid assay in mammalian cells. CV-1 cells were transfected with a reporter plasmid pGAL4-*tk*-Luc and plasmids expressing fusion proteins of Gal4 DBD and NFAT (pG4-NFAT) or RACK-1 and VP16 trans-activation domain (pVP16-RACK) as described in **Materials and Methods**. At 24 h after transfection, cells were harvested and lysed. Luciferase activity was normalized with β-gal activity. Symbol (–) indicates transfection of the reproter plasmid only. Data represent means ± S.E. of three independent experiments. **B.** Coimmunoprecipitation of RACK-1 and NFAT. HA-NFATc.β and myc-RACK-1 were produced individually by using *in vitro* TNT Quick coupled transcription/translation system (Promega). Same volume (20 μl) of each reaction mixture was mixed as indicated and immunoprecipitated with either anti-myc monoclonal antibody (M) or preimmune mouse IgG (G). The immunoprecipitates were analysed by an immunoblot assay with rabbit anti-HA antibody and visulalized by ECL. To assess the amounts of HA-NFATc.β and myc-RACK-1 added to the immunoprecipitation assay, the same volume (20 μl) of *in vitro* translation mixtures were analyzed by an immunoblot assay.

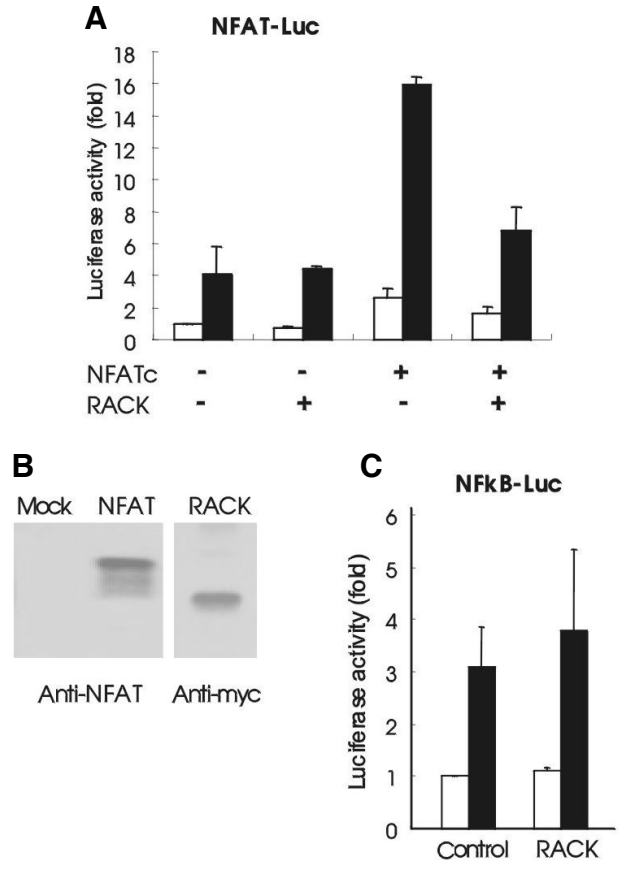

Fig. 2. RACK-1 specifically represses transcriptional activity of NFAT. **A.** CV-1 cells were transfected with pNFAT-Luc, pCH110 and expression vectors of NFATc.β and myc-RACK-1 as indicated. Transfected cells were either unstimulated (open bar) or stimulated (filled bar) with 25 ng/ml PMA and 1 μM ionomycin for 7 h and then assayed for luciferase activity. Luciferase activity was normalized with β-gal activity. **B.** Immunoblot assay of CV-1 cells transfected with NFATc.β or myc-RACK-1 expression vectors. Same number of CV-1 cells were transfected with 3 μg of empty, NFATc.β, or myc-tagged RACK-1 expression vectors, and cell extracts were analyzed by immunoblot assays with an anti-NFAT antibody and an anti-myc monoclonal antibody to prove production of NFAT and RACK-1 proteins, respectively. **C.** CV-1 cells were transfected with p3X-kB-Luc, pCH110 and myc-RACK-1 expression vector. Transfected cells were either unstimulated (open bar) or stimulated (filled bar) with 25 ng/ml PMA and 1 μM ionomycin for 7 h and then assayed for luciferase activity. Luciferase activity was normalized with β-gal activity. Control indicates transfection with an empty expression vector. Data represent means ± S.E. of three independent experiments.

assays, these results demonstrated that RACK1 is indeed physically associated with NFAT proteins in mammalian cells.

Repression of NFAT transcriptional activity by RACK-1 To assess the functional consequence of the interaction, we have tested whether RACK-1 affects transactivation activity of NFAT (Fig. 2A). A reporter construct pNFAT-Luc expressing luciferase driven by three copies of IL-2 NFAT sites upstream of thymidine kinase minimal promoter was transiently transfected with NFAT and RACK-

1 expression vectors into CV-1 cells. In CV-1 cells transfected with pNFAT-Luc alone, stimulation of the cells with PMA and ionomycin induced luciferase activity by 4-fold. We assume that the luciferase induction was caused by endogenous AP-1 activity because of the facts that our immunblot analysis of CV-1 cells indicated no endogenous NFAT expression (Fig. 2B) and also that the IL-2 NFAT site comprises a composite site of NFAT and AP-1 proteins (Jain *et al.*, 1992). Moreover, this induction level of luciferase activity was not affected by RACK-1 expression. On the other hand, cotransfection of NFAT expression vectors augmented luciferase activity by approximately 16-fold, and this additional induction was substantially suppressed by RACK-1 expression. Taken together with the immunoblot analysis confirming production of NFAT and RACK-1 proteins from the transfected expression vectors, these results demonstrate that RACK-1 suppresses transcriptional activity of NFAT.

In addition, to investigate whether RACK-1 affects transactivation activity of other transcription factor such as NF-κB, an NF-κB reporter plasmid p3X-kB-Luc was transiently transfected with either RACK-1 expression or empty vectors into CV-1 cells (Fig. 2C). Stimulation of cells with PMA and ionomycin induced luciferase activity, but this induction was not repressed by RACK-1 expression. Thus, these results indicate that RACK-1 specifically represses NFAT transactivation.

How does RACK-1 inhibit NFAT activity? NFAT activation is primarily regulated in a highly dynamic process of phosphorylation and dephosphorylation. In this process, NFAT kinases and the phosphatase calcineurin play opposite roles in the regulation of NFAT activation, such as subcellular localization and DNA binding activity. Recently, although RACK-1 was originally characterized as a receptor for protein kinase Cβ (PKCβ) that localizes activated PKCs to their sites of action, increasing amounts of evidence demonstrate that RACK-1 interacts with diverse proteins in intracellular signaling processes, including Src kinase, integrin β subunit, cAMP-dependent phosphodiesterase isoform PDE4D5, type I interferon receptor, and Epstein-Barr virus activator protein BZLF1 (Baumann *et al*., 2000; Chang *et al*., 1998; Liliental *et al.*, 1998; Yarwood *et al*., 1999). Moreover, three-dimensional structure of G-protein β subunit, a homolog of RACK-1, revealed a rigid scaffold structure that may serve as an anchor for interacting proteins (Lambright *et al*., 1996). These observations suggest additional PKC-unrelated functions of RACK-1, in which RACK-1 may act as an adaptor or scaffold protein to recruit other proteins into signaling complexes. In this respect, it is possible that RACK-1 bound to NFAT may recruit an NFAT kinase(s) that is yet to be identified, thereby inhibiting NFAT activity by increasing phosphorylation of NFAT. As an alternative mechanism, we are also unable to rule out the possibility that RACK-1 may compete for NFAT binding with calcineurin or other nuclear import receptors, thus preventing NFAT dephosphorylation and nuclear import. Further studies particularly focused on NFAT phosphorylation and subcellular localization will be needed to elucidate the mechanism of NFAT regulation mediated by RACK-1.

Acknowledgments The authors thank A. Rao for providing pGAL4 DBD-E2F; G. Crabtree for an pNFAT-luc plasmid; B. Sugden for a p3X-kB-Luc construct; M.-O. Lee for pGal4-*tk*-Luc and pVP16. This work was supported by Korea Research Foundation Grant (KRF-2000-015-DP0412).

References

Baumann, M., Gires, O., Kolch, W., Mischak, H., Zeidler, R., Pich, D., and Hammerschmidt, W. (2000) The PKC targeting protein RACK1 interacts with the Epstein-Barr virus activator protein BZLF1. *Eur. J. Biochem*. **267**, 3891–3901.

Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997) Nuclear export of NF-Atc enhanced by glycogen synthase kinase-2. *Science* **275**, 1930–1934.

Chang, B. Y., Conroy, K. B., Machleder, E. M., and Cartwright, C. A. (1998) RACK1, a receptor for activated C kinase and a homolog of the b subunit of G proteins, inhibits activity of Src tyrosine kinases and growth of NIH3T3 cells. *Mol. Cell. Biol*. **18**, 3245–3256.

Chow, C. W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R. J. (1997) Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. *Science* **278**, 1638– 1641.

Crabtree, G. R. (1999) Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin, and NF-AT. *Cell* **96**, 611–614.

Garcia-Rodriguez, C. and Rao, A. (1998) Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). *J. Exp. Med*. **187**, 2031–2036.

Jain, J., McCaffrey, P. G., Valge-Archer, V. E., and Rao, A. (1992) Nuclear factor of activated T cells contains Fos and Jun. *Nature* **356**, 801–804.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature* **379**, 311–319.

Liliental, J. and Chang, D. D. (1998) Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. *J. Biol. Chem*. **273**, 2379–2383.

Loh, C., Carew, J., Kim, J., Hogan, P. G., and Rao, A. (1996) T-cell receptor stimulation elicits an early phase of activation and a later phase of deactivation of the transcription factor NFAT1. *Mol. Cell. Biol*. **16**, 3945–3954.

Luo, C., Burgeon, E., and Rao, A. (1996) Mechanisms of transactivation by nuclear factor of activated T cells-1. *J. Exp. Med*. **184**, 141–147.

Mitchell, T. and Sugden, B. (1995) Stimulation of NF-kappaB-mediated transcription by mutant derivatives of the latent membrane protein of Epstein-Barr virus. *J. Viol*. **69**, 2963–

2976.

Mochly-Rosen, D., Khaner, H., and Lopez, J. (1991) Identification of intracellular receptor proteins for activated protein kinase C. *Proc. Natl. Acad. Sci. USA* **88**, 3997–4000.

Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N., and Rosenthal, N. (1999) IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* **400**, 581–585.

Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J. P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P. G., and Rao, A. (2000) Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol. Cell* **6**, 539–550.

Oum, J.-H., Han, J., Myung, H., Hleb, M., Sharma, S., and Park, J. (2002) Molecular mechanism of NFAT family proteins for differential regulation of the IL-2 and TNF-α promoters. *Mol. Cells* **13**, 77–84.

Park, J., Yaseen, N. R., Hogan, P. G., Rao, A., and Sharma, S. (1995) Phosphorylation of the transcription factor NFATp inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. *J. Biol. Chem.* **270**, 20653–20659.

Park, J., Takeuchi, A., and Sharma, S. (1996) Characterization of a new isoform of the NFAT (nuclear factor of activated T cell) gene family member NFATc. *J. Biol. Chem.* **271**, 20914–20921.

Rao, A., Luo, C., and Hogan, P. G. (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707–747.

Ron, D., Chen, C.-H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. *Proc. Natl. Acad. Sci. USA* **91**, 839–843.

Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370–373.

Timmerman, L. A., Clipstone, N. A., Ho, S. N., Northrop, J. P., and Crabtree, G. R. (1996) Rapid shuttling of NF-AT in discrimination of Ca^{2+} signals and immunosuppression. *Nature* **383**, 837–845.

Yarwood, S. J., Steele, M. R., Scotland, G., Houslay, M. D., and Bolger, G. B. (1999) The RACK1 signaling scaffold protein selectively interacts with the camp-specific phosphodiesterase PDE4D5 isoform. *J. Biol. Chem.* **274**, 14909–14917.

Zhu, J. and McKeon, F. (1999) NF-AT activation requires suppression of Crm1-dependent export by calcineurin. *Nature* **398**, 256–260.

Zhu, J., Shibasaki, F., Price, R., Guillemot, J. C., Yano, T., Dotsch, V., Wagner, G., Ferrar, P., and McKeon, F. (1998) Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase 1 and MEKK1. *Cell* **93**, 851–861.