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

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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 communoprecipitation 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 Ca²⁺/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

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 × 10⁵ 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 histocompatibility 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 β-galactosidase (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 104–395) 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
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 immunoblot 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 endogenous NFAT expression. 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.

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References


Inhibition of NFAT Transactivation by RACK-1


