Calcineurin/Nuclear Factors of Activated T Cells (NFAT)-activating and Immune-receptor Tyrosine-based Activation Motif (ITAM)-containing Protein (CNAIP), a Novel ITAM-containing Protein That Activates the Calcineurin/NFAT-signaling Pathway*

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We report in this study the identification and characterization of a novel protein that we designated as calcineurin/NFAT-activating and immune-receptor tyrosine-based activation motif (ITAM)-containing protein (CNAIP). The predicted 270-amino acid sequence contains an N-terminal signal peptide, an immunoglobulin domain in the extracellular region, a transmembrane domain and an ITAM in the cytoplasmic tail. Quantitative reverse transcription-PCR showed that CNAIP was preferentially expressed in neutrophils, monocytes, mast cells, and other immune-related cells. Co-transfection of CNAIP expression constructs with luciferase reporter plasmids in HMC-1 cells resulted in activation of interleukin-13 and tumor necrosis factor-α promoters, which was mediated through the calcineurin/NFAT-signaling pathway. Mutation of either or both tyrosines in the ITAM abolished transcriptional activation induced by CNAIP, indicating that the ITAM is indispensable for CNAIP function in activating cytokine gene promoters. Thus, it is concluded that CNAIP is a novel ITAM-containing protein that activates the calcineurin/NFAT-signaling pathway and the downstream cytokine gene promoters.

It is well recognized that the first level regulation of activation or inhibition of an immune response occurs at the cell surface receptor site (1). The signals sensed by the receptors are relayed through their cytoplasmic signaling modules or adaptor molecules to regulate various cellular activities. The immune-receptor tyrosine-based activation motif (ITAM)† is one of such signal modules present in cytoplasmic tails of many antigen and Fc receptors such as T cell receptor (TCR), B cell receptor (BCR), FceRIβ, and FceRIγ. The consensus of ITAM (YXX(L/I)X6–8YXX(L/I)) has been deduced from sequence analysis of existing ITAM-containing receptors (2). In T cells, following the antigen binding to TCR α and β chain, the two tyrosine residues in the ITAM are phosphorylated by Src-family protein tyrosine kinase, Lck or Fyn (1). The phosphotyrosines, in turn, participate in and physically interact with the signal-amplifying kinase, Syk/ZAP-70, as well as other SH2 domain-containing proteins, which leads to the activation of downstream signaling pathways (1). Analogous events occur in B cells, basophils, mast cells, and other immune cells (3).

Nuclear factors of activated T cells (NFAT) are a family of transcription factors expressed in a diverse cell types of immune systems (4–8). NFAT has been implicated in the activation of mast cells (9, 10), B and T lymphocytes (11, 12), and NK cells (13, 14), and plays a key role in the regulation of transcription of a wide variety of cytokines and cell surface receptors that mediate important immune functions, including interleukin (IL)-2 (15), IL-4 (16), IL-5 (17), IL-13 (18), interferon-γ (19), tumor necrosis factor (TNF)-α (20), and granulocytemacrophage colony-stimulating factor (20), as well as the CD40L (16), and cytotoxic T lymphocyte-associated antigen 4 (21). NFAT is activated by stimulation of receptors coupled to calcium/calcineurin signals, such as the antigen receptors on T and B cells (5, 22), Fc receptors on mast cells and basophils (9), the Fcy receptors on macrophages and NK cells (23), and receptors coupled to heterotrimeric G proteins (24).

During a comprehensive search for novel activating receptors expressed on cell surface using the bioinformatics approach, we identified a human gene that encodes a novel ITAM-containing protein. Expression of this protein in HMC-1 cells activated transcription of IL-13 and TNF-α promoters, which was mediated through the calcineurin/NFAT-signaling pathway. Therefore, the newly identified protein was named calcineurin/NFAT activating and ITAM-containing protein, or CNAIP. We report here its identification, gene structure and stimulatory role for NFAT.

EXPERIMENTAL PROCEDURES

Identification and in Silico Characterization of CNAIP—The protein data base compiled by International Protein Index (IPI) (www.ensembl.org/IPI) containing ~65,000 protein sequences (as of March 2002) was used as data source in this study. A hidden Markov model (HMM)-based method was employed for Ig-domain search against IPI data base. The HMM, which was built from an alignment of 113 confident Ig domains and calibrated using the program HMMER, was obtained from the Pfam (version 6.6) data base (25). To identify ITAM-containing proteins, a PROSITE-formatted motif profile was first constructed based on the common features of ITAM motif, and the software “seedtop” (NCBI) was used to perform the search. Large-scale transmembrane region prediction for all of the IPI proteins was carried out by using software TMHMM version 2.0 (26) (www.cbs.dtu.dk/services/TMHMM/). The three sets of genes gen-

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§ The abbreviations used are: ITAM, immune-receptor tyrosine-based activation motif; AP-1, activator protein 1; BCR, B cell receptor; Ig, immunoglobulin; HMM, hidden Markov model; NFAT, nuclear factors of activated T cells; CNAIP, calcineurin and NFAT-activating and ITAM-containing protein; TCR, T cell receptor; IL, interleukin; TNF-α, tumor necrosis factor-α; RT, reverse transcription; luc, luciferase; FACS, fluorescence-activated cell sorter; TREM, triggering receptor expressed on myeloid cells molecule; contig, group of overlapping clones; Cₜ, threshold cycle.

* The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AY247409.
CNAIP comprises six exons (Fig. 1A). Sequence similarity searches against various public databases showed that CNAIP does not share statistically significant similarity with any known protein. Amino acid sequence analysis revealed that CNAIP contains a putative signal peptide sequence (amino acids 1–29), a conserved cysteine-rich domain (amino acids 30–110), the Ig-domain most likely to be a type I transmembrane protein, which contains a putative N-glycosylation site found in the extracellular region, a fat-cyl domain (amino acids 110–150) in the extracellular region, a FAT-a activating and a calculated molecular mass of ~30 kDa. It is predicted to be a type I transmembrane protein, which contains a putative signal peptide sequence (amino acids 1–29), an Ig-domain (amino acids 50–150) in the extracellular region, a transmembrane domain (amino acids 164–186), and an ITAM (amino acids 220–235) in the cytoplasmic region (Fig. 1B). One potential N-glycosylation site was found in the extracellular region (amino acids 107–110). The Ig-domain most likely adopts a V-type fold based on 3D-PSSM fold recognition algorithm (30, 36). CNAIP has been mapped to chromosome 22q13.2 by sequence similarity search. Alignment of cDNA with genomic sequence showed that the coding region of CNAIP comprises six exons (Fig. 1C). Sequence similarity search against various public data bases showed that CNAIP does not share statistically significant similarity with any known proteins.

**Tissue Distribution of CNAIP**—The CNAIP mRNA expres-
tion levels in a number of human cells and tissues were as-

sessed using real time quantitative RT-PCR. The results

showed that CNAIP was highly expressed in neutrophils,

primary monocytes and monocytic cell lines (THP-1), lympho-

cytes, and in vitro cultured mast cells derived from cord blood

(Fig. 2). The expression in spleen and lung was also evident. In

contrast, the CNAIP expression in all other tissues and cells

(brain, heart, kidney, liver, Daudi, HPB-ALL, Jurkat, HMC-1,

and HUVAC) was low, suggesting that the primary role of

CNAIP may be restricted to the immune system. Interestingly,

the CNAIP expression level in more mature mast cells (cul-
tured for 7 weeks and 86% tryptase-stained positive) is about

3-fold higher than that in the earlier stage of mast cells (cul-
tured for 4 weeks and 17% tryptase-stained positive).

Expression and Subcellular Localization of CNAIP Pro-

tein—To characterize the CNAIP gene product, we made two

expression constructs, one with a V5 tag fused in frame to the

C terminus of CNAIP coding region (CNAIP-V5C) and the

other with the native signal peptide replaced by a heterologous

signal peptide in the vector, which is immediately followed by

a V5 tag fused to the N-terminal region of the truncated CNAIP

(CNAIP-V5N). Transient transfection of CNAIP-V5C into 293T

cells showed two protein bands of 33 and 36 kDa in Western

blot, which are absent in cells transfected with the empty

vector (data not shown). In protein fractions prepared by

freeze-thaw cycle and high-speed centrifugation, the 33 and 36

kDa proteins were only detected in the insoluble membrane

fraction, not in the soluble cytosol fraction (Fig. 3), indicating

that CNAIP is a membrane-associated protein.

Immunofluorescence staining was then performed to further
determine the subcellular localization and the orientation of

CNAIP in the membrane. FACS analysis of 293T cells

transfected with CNAIP-V5N or CNAIP-V5C showed that N termi-
nus-tagged CNAIP was detected only in the living cells trans-

fected with CNAIP-V5N (Fig. 4) but not in the living cells

transfected with CNAIP-V5C (data not shown). However, fix-
ation of the cells transfected with the two expression constructs
resulted in unambiguous detection of both the N terminus- and

C terminus-tagged protein by FACS and fluorescent micros-
copy (data not shown). These results indicated that CNAIP is a
transmembrane protein with the N terminus exposed to the
outside of the cellular membrane.

CNAIP Activates the Calcineurin/NFAT-signaling Pathway—
The co-existence of Ig domain and ITAM motif in CNAIP, along
with the preferential expression in immune cells, strongly sug-

gest that CNAIP may function as an activating receptor in im-
mune system. To test if CNAIP can activate the transcription of
cytokine genes, we co-transfected CNAIP-V5N with luciferase
reporter constructs that were linked to IL-13, TNF-α, or FcεRIα
promoter in HMC-1 cells. The luciferase reporter assays showed
that CNAIP increased IL-13 and TNF-α promoter activities by
−14- and −5-fold, respectively, as compared with the expression

FIG. 1. CNAIP amino acid sequence, ITAM alignment, and
gene structure. A, protein sequence of CNAIP. The putative signal
peptide and transmembrane region were indicated by single and double
underlines, respectively, and the ITAM was shaded. B, alignment of
ITAM sequences of selected human proteins. C, schematic representa-
ction of CNAIP gene structure.
vector control. In contrast, the FcRIα promoter-linked luciferase activity was not changed (Fig. 5A).

It was well documented that NFAT is involved in transcriptional activation of cytokine genes, such as IL-13 (18) and TNF-α (20), and that ITAM-containing receptors, upon ligand binding, can lead to the activation of several transcription factors including NFAT (37), NF-κB (38), and AP-1 (39). To test whether NFAT, NF-κB, and/or AP-1 are potential downstream targets of the CNAIP-signaling pathway, we assessed the NFAT, NF-κB, or AP-1 luciferase reporter activity by transient transfection with CNAIP-V5N in HMC-1 cells. Expression of CNAIP elevated NFAT luciferase reporter activity by 19-fold as compared with the transfection with the vector only, whereas the NF-κB or AP-1 luciferase reporter activities showed no significant changes (Fig. 5A). To confirm that full-length CNAIP with its native signal peptide can also activate NFAT, we co-expressed a full-length construct of CNAIP with an NFAT luciferase reporter. Expression of full-length CNAIP also elevated NFAT luciferase reporter by 9-fold as compared with the transfection with a control vector (Fig. 5C). These data were reproducible in three separate experiments.

Because calcium flux and calcineurin activation are signaling events upstream of NFAT activation (12), we tested whether the CNAIP/calcineurin/NFAT-signaling pathway could be blocked by cyclosporin A, an immune-suppressive agent which specifically inhibits calcineurin activation. Addition of 1 μM of cyclosporin A to the culture inhibited CNAIP-mediated NFAT activation by 90% (Fig. 6), indicating that CNAIP can activate calcineurin/NFAT-mediated signaling cascade and therefore activate transcription of NFAT-regulated cytokine genes.

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Transfection of either of these mutants into HMC-1 cells failed to activate IL-13, TNF-α, or NFAT luciferase reporter activity (Fig. 5). The relative luciferase reporter activity in these cells was comparable with that of cells transfected with vector control (Fig. 5A). Western blot analysis showed that mutant proteins were expressed at similar level as that of wild type CNAIP (Fig. 5B), indicating that mutations do not affect the expression and stability of CNAIP in these cells. These data indicated that activation of the calcineurin/NFAT-signaling pathway by CNAIP is mediated by ITAM, and both tyrosine residues are required for the ITAM-mediated function in CNAIP.

DISCUSSION

In the present report, we describe the identification of a novel ITAM-containing protein, CNAIP, that activates NFAT, IL-13, and TNF-α promoter activity. Our data suggest the CNAIP may function as an activating receptor. ITAM-containing receptors are a divergent group of cell surface membrane proteins that are expressed in immune-related cells and regulate cell growth, maturation, apoptosis, and cell activation. Members of this family include Igα, Igβ, TCR-ε, TCR-γ, CD3γ, CD3δ, CD3ε, FceRIβ, and FcεRIγ. Some of them contain Ig or Ig-like domains in the extracellular region, which are involved in protein-protein interaction. Similar to those receptors, CNAIP contains an Ig domain in the extracellular region. Some ITAM-containing receptors transduce signal within a multisubunit of immune receptor complexes such as TCR and BCR. It is not clear at present whether CNAIP is a component of an immune receptor complex or functions as a single unit. Interestingly, overexpression of CNAIP can activate downstream effectors without ligand binding or antibody cross-linking. The mechanisms by which CNAIP activates the signaling pathway remain to be determined. However, it is possible that overexpression of CNAIP proteins in the cells results in the aggregation or clustering of the receptors on cell surface, which leads to the recruitment of downstream effector to the receptor molecules that subsequently lead to its activation.

The ITAM consensus sequence (YxxL/Ix 6YxxL/I) contains a critical role in acute inflammatory responses to bacteria (41). It is conceivable that CNAIP may transduce its activating molecules such as NKp44 and TREMs (triggering receptor expressed on myeloid cells molecules). All these molecules are type I transmembrane protein with a single Ig domain in the extracellular region, and transduce signals via ITAM motif, which is contained in either the cytoplasmic region of the molecule or its associated adaptor molecule DAP12. Moreover, both TREMs and CNAIP are abundantly expressed in neutrophils and monocytes. However, sequence similarity between CNAIP and TREM and NKP44 is very low (<10%) and statistically insignificant. TREM-1 was recently characterized and found to function as an activating receptor (41). It is expressed at high levels on neutrophils and monocytes that infiltrate human tissues infected with bacteria and plays a critical role in acute inflammatory responses to bacteria (41). It is tempting to speculate that CNAIP may play similar roles in acute inflammation, which are characterized by an exudate of neutrophils and monocytes (42). Further studies are aimed to identify the functional role of CNAIP in the immune responses.

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