Truncation of C-mip (Tc-mip), a New Proximal Signaling Protein, Induces c-maf Th2 Transcription Factor and Cytoskeleton Reorganization

Philippe Grimbert,[1] Asta Valanciute,[1] Vincent Audard,[1] André Pawlak,[1] Sabine Le gouvelo,[1] Philippe Lang,[2] Patrick Niaudet,[3] Albert Bensman,[4] Georges Guellaën,[1] and Djillali Sahali[1]

[1]*Unité INSERM 99, and* [2]*Service de néphrologie adulte, Hôpital Henri Mondor, AP-HP, Université Paris XII, 94010 Créteil, France*

[3]*Service de néphrologie, Hôpital Necker-Enfants Malades, AP-HP, 75015 Paris, France*

[4]*Service de néphrologie, Hôpital Armand Trousseau, AP-HP, 75012 Paris, France*

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Abstract

Several arguments suggest that minimal change nephrotic syndrome (MCNS) results from yet unknown systemic disorder of T cell function. By screening a cDNA library from T cell relapse, we identified a new pleckstrin homology (PH) domain-containing protein encoded by a gene located on chromosome 16q24. Two alternative transcripts were identified. The first species (c-mip) was expressed in fetal liver, kidney, and peripheral blood mononuclear cells (PBMCs), but weakly detected in PBMCs from MCNS patients. The second form (Tc-mip, standing for truncated c-maf inducing protein), corresponds to subtracted transcript and lacks the NH_2-terminal PH domain. The expression of Tc-mip was restricted to fetal liver, thymus, and MCNS PBMCs where it was specifically recruited in $CD4^+$ T cells subset. Overexpression of Tc-mip in T cell Jurkat induced c-maf, transactivated the interleukin 4 gene and down-regulated the interferon γ expression, characteristic of a Th2 commitment. Moreover, the overexpression of Tc-mip induced Src phosphorylation, T cell clustering, and a cellular redistribution of the cytoskeleton-associated L-plastin, by a PI3 kinase independent pathway. Tc-mip represents therefore the first identified protein, which links proximal signaling to c-maf induction.

Key words: lipoid nephrosis • T lymphocytes • pleckstrin domain • signal transduction

Introduction

Minimal change nephrotic syndrome (MCNS) is the most frequent glomerular disease in children, characterized by heavy proteinuria with relapse and remission courses (1). Although no immune cell infiltration, or immune complex deposit could be identified in the kidney, many arguments suggest that MCNS results from a systemic disorder of T cell function (2). Indeed, relapses often occur in the context of immune challenge initiated by infectious, allergic stimuli or vaccine and they are usually sensitive to drugs known to inhibit the immune system (glucocorticoids, cyclosporine, and cyclophosphamide). Nonetheless, the link between immune disorders and this glomerular disease remains unresolved.

Recently, we have shown that T lymphocytes from MCNS were rather driven toward a Th2 phenotype. Supporting this view, we found that T cells display a down-regulation of the IL-12 receptor β2 subunit (IL-12R β2), whereas the second component of the IL-12R, the β1 chain, was normally expressed (3). The IL-12R β2 is selectively expressed by Th1 cells and plays a key role in the transduction of IL-12 signaling through the Jak/Stat pathway. The down-regulation of the IL-12R β2 is compatible with a lack of IL-12 production during relapse (4). The commitment of MCNS T cells along the Th2 pathway involves the recruitment of c-maf of which the functional expression appears closely related to MCNS activity (unpublished data). It has been shown that c-maf promotes T helper cell

Address correspondence to D. Sahali, Unité INSERM 99, Hôpital Henri Mondor, 51, avenue du Mal de Lattre-de-Tassigny, 94010 Créteil, France. Phone: 33-1-49 81 35 30; Fax: 33-1-48 98 09 08; email: sahali@im3.inserm.fr

Abbreviations used in this paper: MCNS, minimal change nephrotic syndrome; PKC, protein kinase C; Tc-mip, truncated c-maf inducing protein.

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type 2 (Th2) and attenuates Th1 differentiation (5, 6). As a matter of fact, patients with MCNS are often unable to mount an effective Th1 response that might account for observed defects in delayed-type hypersensitivity response and recall response to antigens (2, 7).

To understand in depth the molecular mechanisms involved in this T cell dysfunction, we recently identified by subtractive cloning and differential screening, transcripts up-regulated during the active phase of the disease (3). Among them, we isolated a truncated form of a new protein, involved in c-maf signaling pathway, that we named Tc-mip (for truncated c-maf inducing protein). The natural transcript, c-mip, corresponds to the product of the Kiaa1694 gene, previously identified in the human brain (8). Tc-mip exhibited a deletion within the NH_2-terminal pleckstrin homology domain (PH) of c-mip and its transcript is selectively induced in active phase of the disease whereas it is hardly detected in T cells of normal subjects. We show here that overexpression of Tc-mip in Jurkat T cells, strongly induces the c-maf protein and increases the IL-4 promoter-mediated transcription, whereas it concomitantly represses the IFN-γ expression. Moreover, overexpression of Tc-mip in Jurkat T cells, induces T cell clustering and cellular redistribution of L-plastin, a cytoskeleton-associated protein, by a PI3 kinase–independent pathway.

These results suggest that Tc-mip plays a critical role in Th2 signaling pathway and represents the first proximal signaling protein which links TCR-mediated signal to the activation of c-maf Th2 specific factor.

Materials and Methods

Patients. The cohort of patients analyzed in this study has been described previously (3, 9). In children, the criteria of the International Study of Kidney Diseases were used for diagnosis and management of MCNS (10). In adults, the diagnosis of MCNS or Membranous Nephropathy (MN) was confirmed by renal biopsy before inclusion. All patients with relapse (children and adults) had proteinuria over 3 g/24 h, and low serum albumin levels (below 3 g/dl), at the time of blood sampling, which was performed before the beginning of steroid treatment.

Remission samples were collected during periods of inactive disease, defined by a proteinuria below 0.2 g/24 h. Controls consisted of normal children studied while undergoing routine analysis, normal adult volunteers, as well as patients with MN.

Informed consent was obtained from the parents and whenever possible from the pediatric patients, as well as from adult patients and normal volunteers.

Antibodies and Reagents. Immunoselection of T cell subsets and monocytes was performed using a cocktail of hapten-conjugated antibodies (Miltenyi Biotech).

A polyclonal antibody common to c-mip and Tc-mip was produced against a peptide of 15 aa, corresponding to amino acids 194–209 of the c-mip/Tc-mip (Eurogentec). Antibodies against c-maf (sc-7866) and phospho Akt (ser-473) were purchased from Santa Cruz Biotechnology Inc. and New England Biolabs, Inc., respectively. Monoclonal antibody of L-Plastin (Anti-LPLA4.1) was a gift of Drs. Eric J. Brown and Hua Shen (University of California, San Francisco, CA) and was used at 1 μg/ml. Wortmanin was purchased from Sigma-Aldrich). Anti-phospho Src family (Tyr416) antibody was purchased from New England Biolabs, Inc.

Purification of PBMC and T Cell Subsets. PBMCs and T cell subsets were purified as described previously (9). The purity of the preparations was 88–96%, as assessed by flow cytometric analysis, using FITC-conjugated CD2, $CD4^+$, CD19, and CD8 antibodies. Activation was performed with 5 μg/ml PMA (phorbol ester myristate) and 5 μg/ml calcium ionophore (ionomycin), for 4 h. Th1 and Th2 polarized T cells were a gift of Dr. Pellegrini (Institut Pasteur, Paris, France).

Immunocytochemistry. Cells were cytospun at 10^5 cells/slide, fixed and permeabilized by using methanol at –20°C, then processed for immunoreactivity. Cells were incubated in the blocking solution (10% normal sheep serum (NSS), 1% BSA for 40 min, washed twice with PBS, then incubated with Tc-mip (1/200) or L-plastin (1 μg/ml) antibody (in 5% NSS, 1% BSA, 0.1% Tween 20) for 2 h at room temperature. Slides were washed three times with PBS, then incubated with anti–rabbit Cy3-labeled antibody (1/1,000 in blocking solution) for 30 min. Slides were mounted in a Vectashield DAPI (Vector Laboratories), and analyzed on an Axioplan Zeiss microscope equipped for epifluorescence. The percentage of positive cells was determined on an average of 200 cells.

RT-PCR. Total RNAs were treated by DNase I and purified using Rneasy kit (QIAGEN), following the supplier's protocol. The 5′ and 3′ primers of Tc-mip and c-mip were selected in the first respective exon. The sequence of the primers, and PCR parameters are indicated in Table I.

Semiquantitative RT-PCR was performed as previously reported (9). Each cycle consists of denaturation at 94°C for 30 s, annealing at the indicated temperature for 30 s and extension at 68°C for 2 min. Amplified products were detected on Southern blots with [^{32}P] labeled specific internal oligonucleotide probes and quantified by using a PhosphorImager (Storm 840, Molecular Dynamics SA), coupled to the ImageQuant v1.11 analysis software. PCR reactions were normalized for GAPDH expression.

Northern Blot. Human multiple-tissue Northern blots (CLONTECH Laboratories, Inc.) were hybridized with a 560 bp cDNA probe common to c-mip and Tc-mip or with a Tc-mip specific probe corresponding to the first exon of Tc-mip. Hybridization and blot processing were performed as described previously (3).

DNA Sequencing. Preparation and sequencing of double stranded plasmid DNA template and sequencing were performed as reported previously (3). Nucleic acid and protein database searches were done using resources of the National Center Biotechnology Information.

Western Blots and Electromobility Shift Assays. Preparation and quantification of protein extracts, as well as SDS-PAGE immunoblotting, and electromobility shift assay (EMSA) experiments, were performed as described previously (9). The double-stranded oligonucleotide probes (100 ng; Genset), consisting of the wild (5′-GGAATTGCTGACTCAGCATTACT-3′) and the mutant (5′-GGAATTGCTGACTCATTGTTACT-3′) MAREs containing the c-Maf recognition sequence (underlined). The specificity of band shift was also tested by preincubation of nuclear extracts with 2 μg of polyclonal antibodies raised against c-maf.

Cloning of the 5′ End of c-mip by Rapid Amplification of cDNA Ends (RACE). Additional sequence upstream of the 5′end of the c-mip mRNA was explored by using the 5′/3′RACE kit (CLONTECH Laboratories, Inc.) and a universal antisense primer included in the kit and a 28 mer antisense oligonucleotide located at the position 145–172 of the c-mip-exon 1 (5′-GCT-

Table I. *Sets of Primers Used in Semiquantitative RT-PCR and for PCR Amplification of Coding Sequences of c-mip and Tc-mip*

mRNA	Oligonucleotides	Accession no.	Expected size (bp)	Tm annealing (°C)	PCR cycles
GAPDH	S: 5′-ACCACAGTCCATGCCATCAC-3′	NM 004048	374	58	25
	AS: 5′-TCCACCACCCTGTTGCTGTA-3′				
	I: 5′-CTCAAGGGCATCCTGGGCTACACTGAGCAC-3′				
c-maf	S: 5′-TGCACTTCGACGACCGCTTCTC-3′	AF055376	326	62	32
	AS: 5′-CGCTGCTCGAGCCGTTTTCTC-3′				
Tc-mip	S: 5′-CCTGCGAGGAGGGAAGTTACAGATCTC-3′	AY172689			
	AS: 5′-CAGGTAGCTATTGGCAGCCTGCAGTAAG-3′		209	62	32
	I: 5′-ATGGGACAGGCTGCTGAGCCAACT-3′				
c-mip	S: 5′-GAAGGCACGAAGATGGGCGCCGTGCCCT-3′	ABO51481	130	75	32
	AS:5′-GCTGAGAAAGGTCCGCGGGTGCCGGATG-3′				
IL-4	S:5′-TTCTCCTGATAAACTAATTGCCTCACATTGTC-3′	XM004053	143	60	34
	AS:5′-GGTGATATCGCACTTGTGTCCGTGG-3′				
IFN-γ	S: 5′-GGTTCTCTTGGCTGTTACTGC-3′	XM006883	294	60	34
	AS: 5′-GTCATCTCGTTTCTTTTTGTTGCT-3′				
Tc-mip (CS)	S: 5′-ATGGGACAGGCTGCTGAGCCAA-3′	AY172689	2150	63	32
	AS: 5′-CTTCCTGCCTTGAGCTGGGAGC-3′				
c-mip (CS)	S: 5′-ATGGGCGCCGTGCCCT-3′	ABO51481	2270	56	32
	AS: 5′-CTTCCTGCCTTGAGCTGGGAGC-3′				

The oligonucleotides sense (S) and antisense (AS) were selected from the sequences with the indicated accession numbers. The size of each amplified product, annealing temperature of each oligonucleotide, and the number of PCR cycles are indicated. I, internal oligonucleotide probe; CS, coding sequence.

GAGAAAGGTCCGCGGGTGCCGGATG-3′). The PCR product was blunt ended and cloned into Topo Zero blunt end vector (Invitrogen), according to manufacturer's protocol. Preparation and sequencing of double stranded plasmid DNA template and sequencing were performed as described previously (3).

Plasmids Construction. The cDNA corresponding to the coding sequences of Tc-mip and c-mip were obtained by RT-PCR using PBMC RNA from a patient with MCNS relapse and a normal subject, respectively. The specific primers are reported in Table I. The amplified products were subcloned into pcDNA3.1/V5-His TOPO cloning vector (Invitrogen). Chloramphenicol acetyltransferase (CAT)-based human IL-4T promoter construct was a gift of Dr. V. Casolaro (Johns Hopkins University School of Medicine; reference 11).

Transient Transfections. Jurkat T cells were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 supplemented with 10% FCS. Transient transfections were performed by electroporation of 30×10^6 cells with 30 μg of each plasmid (Tc-mip, c-mip, or empty vector), or in combination with 10 μg of CAT-IL4-reporter construct, using a Bio-Rad Laboratories Gene Pulser set at 250V and 960 μF. The transfected cells were allowed to recover overnight, then were divided into identical aliquots in 24-well plates. One aliquot was left unstimulated whereas the other was stimulated with OKT3 ascite fluid (1:5,000) for 6 h. In some experiments, wortmanin (100 nM) was added to the culture medium 6 h following transfection and left overnight. CAT assays were performed according to the manufacturer's protocol (Promega). CAT activities, normalized by protein content, were determined using a liquid scintillation counting assay (Packard Instrument Co.), and were shown as fold induction relative to control, between stimulated and unstimulated conditions. Efficiency of transfection was controlled by Western blotting of cytosolic extracts, using the anti-c-mip/Tc-mip antibody.

Results

1-Identification and Characterization of Tc-mip. A Tc-mip cDNA clone (4.1kb, GenBank/EMBL/DDBJ accession no.: AY172689) was isolated by differential screening of a full length cDNA library, generated from T cell-enriched PBMCs of MCNS relapse, using subtracted probes from relapse versus remission (unpublished data). The Tc-mip mRNA corresponds to an alternative species of c-mip (4.2 kb), isolated from human brain (8). Both Tc-mip and c-mip are transcribed from a single gene comprising 21 exons spanning 268 Kb in length and located on chromosome 16q24 (Fig. 1). A Tc-mip 5′ RACE did not reveal upstream 5′ sequence in c-mip and Tc-mip (data not depicted), suggesting that both mRNA are transcribed from distinct promoter regions.

The Tc-mip and c-mip cDNAs contain an open reading frame of 2040 and 2220 nucleotides, encoding 679 (75 kD) and 739 (85 kD) amino acid proteins respectively. Both initiation codons (ATG) are flanked by the Kozak consensus sequence. The c-mip protein contains a pleckstrin homology (PH) domain encoded by exons 1, 2, 3, and 4. The

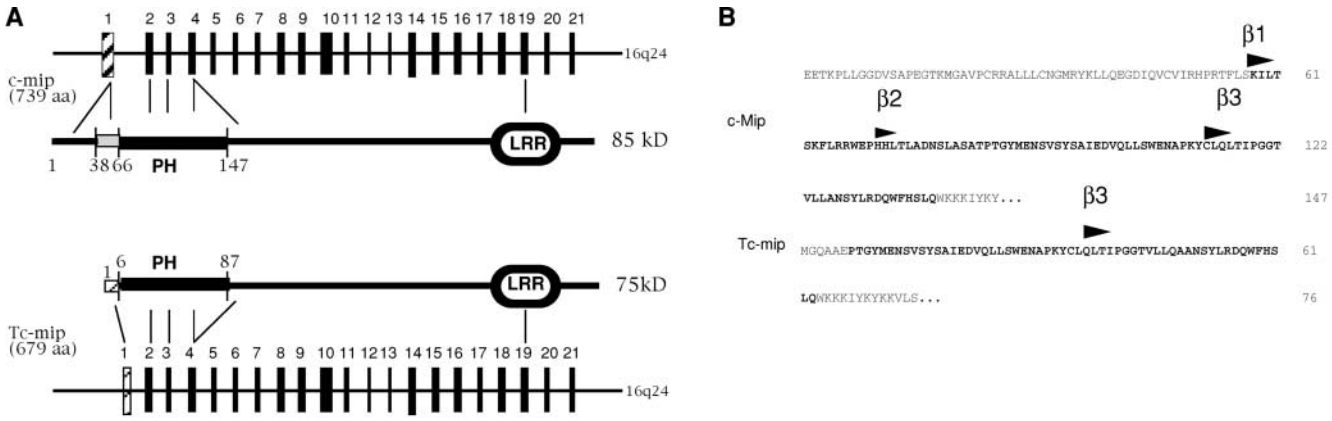

Figure 1. Comparative structure of c-mip and Tc-mip. (A) Genomic structure of c-mip and Tc-mip. Exons are depicted as numbered boxes and introns are not drawn to scale. Dashed boxes indicate identical exons. Between gene structures are representation of transcript products. Protein structure was analyzed by Smart software (Simple Modular Architecture Research Tool), which detected the PH domain (encoded by exons 1, 2, 3, and 4 and the Leucine-rich repeats (LRR), encoded by the exon 19). Both transcripts differ by use of the first exon indicated by a striped box: c-mip exon 1 encodes for 66 amino acid (aa) of which the 29 aa COOH-terminal (position 38–66) contribute to PH domain (109 aa); Tc-mip exon-1 encodes for 6 aa outside of the PH domain (80 aa). (B) Prediction of β sheet structures of the PH domain using the Garnier Peptide structure tools (http://biotools.umassed.edu/cgi-bin/biobin/garnier). Amino acid sequence corresponding to the PH domain is indicated in bold characters.

PH domain starts at position 38 of exon 1 that encodes 66 amino acids. The Tc-mip transcript does not contain the first exon of c-mip but starts with a new 145 nucleotide-exon, encoding 6 amino acids and which is located 50 Kb downstream of c-mip-exon 1 on the genome (Fig. 1 A). Thus, Tc-mip exhibits a different amino-terminal part, as compared with c-mip, characterized by an NH_2-terminal truncation of 29 aa within the pleckstrin homology (PH) domain. This change does not modify the reading frame of the remainder of the protein that is identical in both proteins but alters the spatial configuration of the PH domain by deletion of the β sheet 1, the β sheet 2, and the loop β1β2 (Fig. 1 B). Beside the PH domain, Tc-mip and c-mip proteins contain a leucine-rich repeat (LRR) domain encoded by the exon 19. The remainder of the protein does not exhibit significant homology with others known domains but contains several patterns, including protein kinase C (PKC), casein kinase II, and tyrosine, potential phosphorylation sites. The truncation of Tc-mip removes the putative Thr 61, Ser 62, and Lys 63 PKC phosphorylation sites as compared with c-mip.

We hybridized multiple-tissue Northern blots with a cDNA probe common to Tc-mip and c-mip (Fig. 2, top panel). We only detected a signal at 4.25 kb corresponding to c-mip mRNA. The transcript was highly expressed in PBMCs, kidney, and fetal liver and to a lesser extent in adult brain and liver. On the other hand, the Tc-mip specific probe (corresponding to proximal 5′-145 nucleotides of Tc-mip) only hybridized with mRNA originating from thymus and fetal liver (Fig. 2, bottom panel). We were not able to make a Northern blot of Tc-mip transcript in PBMC of MCNS patients because of the scarcity of the biological material.

2-Expression of Tc-mip in Resting and Activated T Cells. As Northern-blot analyses revealed a basal expression of c-mip in PBMCs, we analyzed by semiquantitative RT-

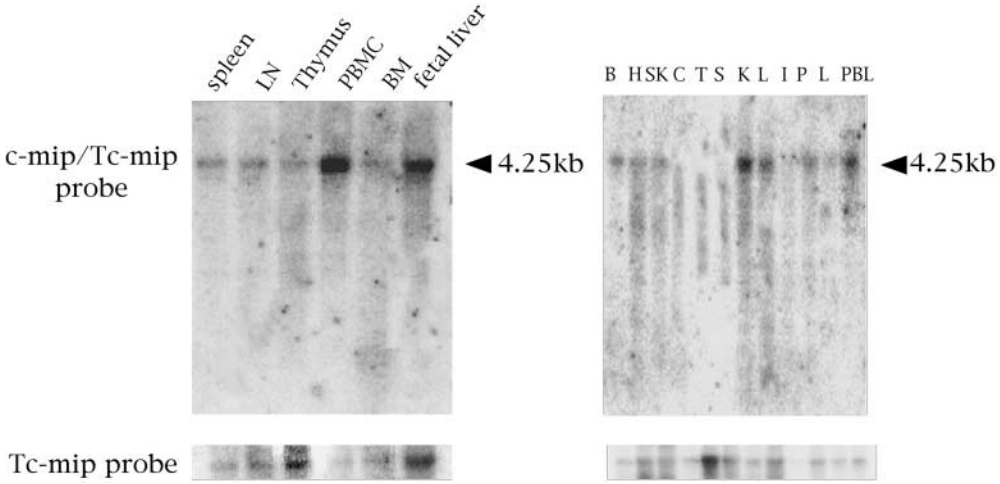

Figure 2. Northern blot analysis of c-mip and Tc-mip. (Top panel) Multiple tissue-Northern blots (CLONTECH Laboratories, Inc.) were hybridized with a 560-bp cDNA probe common to Tc-Mip and c-Mip and exposed on a PhosphorImager Storm for 24 h. BM, bone marrow; B, brain; H, heart; SK, skeletal muscle; C, colon; T, thymus; S, spleen; K, kidney; L, liver; I, intestine; P, placenta; Lu, lung. (Bottom panel) Expression pattern of Tc-mip, using cDNA probe encoded by the Tc-mip exon-1.

PCR, the relative expression of Tc-mip and c-mip mRNA in PBMC subsets purified by immunomagnetic selection. In resting cells, we found that the c-mip transcript was expressed in monocytes, $CD8^+$, $CD4^+$ T cells, and B lymphocytes, whereas the Tc-mip transcript was detected at lower levels only in $CD4^+$ and $CD8^+$ T cells (Fig. 3 A). To determine whether these transcripts are induced in response to activating conditions, cells were exposed to phorbol ester myristate (PMA) and calcium ionophore (ionomycin), for 4 h. The expression of c-mip and Tc-mip mRNAs was markedly down-regulated in activated cells (Fig. 3 A). Then we analyzed the protein expression by immunoblotting with a polyclonal antibody recognizing the amino acid sequence 194–209 common to Tc-mip and c-mip proteins. Contrasting with their basal mRNA expression, we were unable to detect Tc-mip by immunoblotting, whereas c-mip was very faintly or not detected, either in resting or in activated cells (unpublished data), suggesting post transcriptional regulation mechanisms. As the expression of c-mip and Tc-mip felt in normal T cells activated by PMA and CA^{2+}ionophore (Fig. 3 A), we sought to determine whether such effects could be also observed in T cells from MCNS relapse. T cells from relapse were cultured in the presence of PMA/ionomycin and the expression of Tc-mip was analyzed by RT-PCR (Fig. 3 B). In contrast to normal T cells, the expression of Tc-mip in MCNS T cells was not altered by PMA/ionomycin stimulation, suggesting that Tc-mip is involved in some signaling pathway that might be dysregulated in MCNS.

Our recent investigations argue for an early commitment of MCNS T cells along a Th2 phenotype (3). The identification of a new gene up-regulated in this disease led us to investigate its expression in polarized T cells along Th1 or Th2 pathway. Under Th1 skewing conditions (T cells cultured in the presence of IL-12 and anti–IL-4 antibody), the expression of Tc-mip at both mRNA and protein levels was little influenced, relatively to basal expression in resting T cells (Fig. 3 C). By contrast, T cells incubated in the presence of IL-4 and anti–IL-12 antibody (Th2 conditions) exhibited a strong induction of Tc-mip. This result constitutes an additional element strengthening the Th2 bias in MCNS.

3-Specific Induction of Tc-mip in MCNS. We analyzed by semi-quantitative RT-PCR the expression level of c-mip and Tc-mip transcripts during the relapse and the remission phases in nine patients who experienced a first episode of MCNS. The expression of Tc-mip mRNA was increased during the relapse in the nine patients tested, as compared with remission, whereas the eight normal subjects exhibited very low levels of this transcript (Fig. 4 A). In contrast, the expression level of the c-mip transcript was very modest in both relapse and remission phases, whereas it was highly expressed in normal subjects. The up-regulation of Tc-mip mRNA appeared specific to MCNS since four patients with membranous nephropathy, and a similar range of proteinuria, exhibited a Tc-mip and c-mip mRNA levels comparable to normal subjects.

We further studied whether the induction of Tc-mip was restricted to a cell subset during the active phase of the

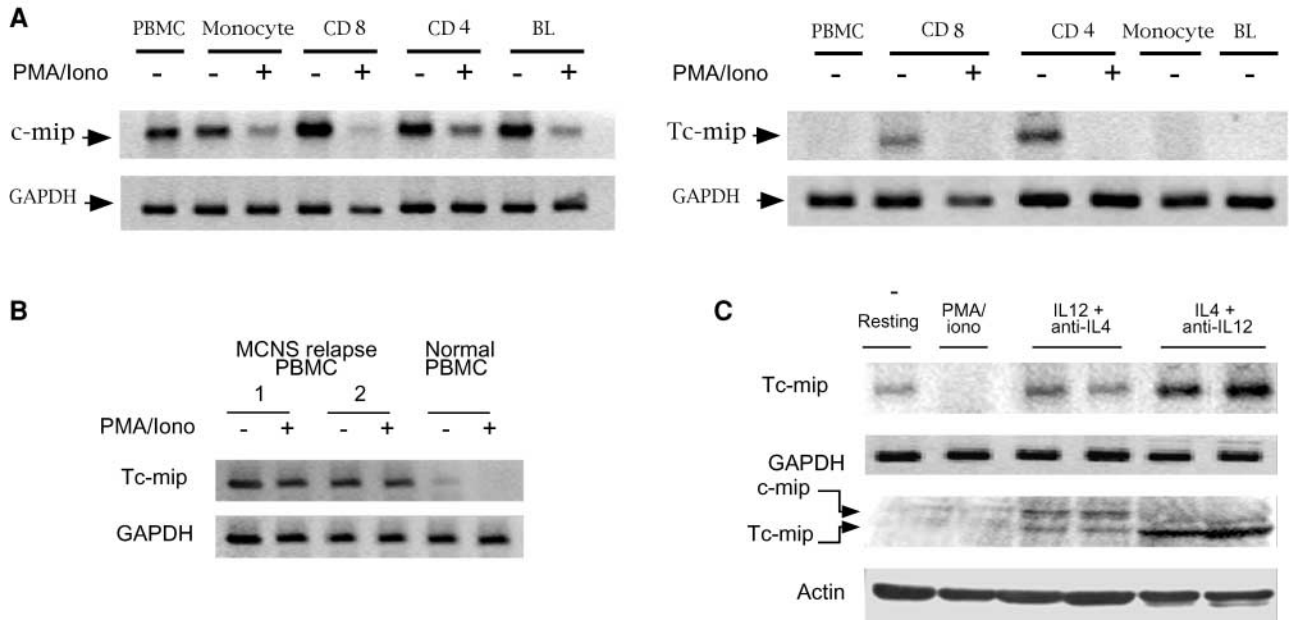

Figure 3. Differential expression of c-mip and Tc-mip in PBMC subsets. (A) Down-regulation of c-mip and Tc-mip by PMA/ionomycin. Resting (−) and PMA/ionomycin-activated (+) cells were isolated and analyzed for c-mip and Tc-mip transcript expression as described under Materials and Methods. The expression of GAPDH was monitored in parallel. PBMC, peripheral blood mononuclear cells. BL, B lymphocyte. (B) Lack of down-regulation of Tc-mip in MCNS T cells activated by PMA/ionomycin. MCNS T cells were activated as described above and then processed for Tc-mip expression by semiquantitative RT-PCR. (C) Preferential induction of Tc-mip in Th2. Naive T cells were purified from blood umbilical cord, cultured in the presence of PMA/Iono, or polarized under Th1 (IL12 + anti-IL4) or Th2 (IL4 + anti-IL12) skewing conditions and analyzed for c-mip/Tc-mip and IL-4 mRNA expression by semiquantitative RT-PCR as described under Materials and Methods.

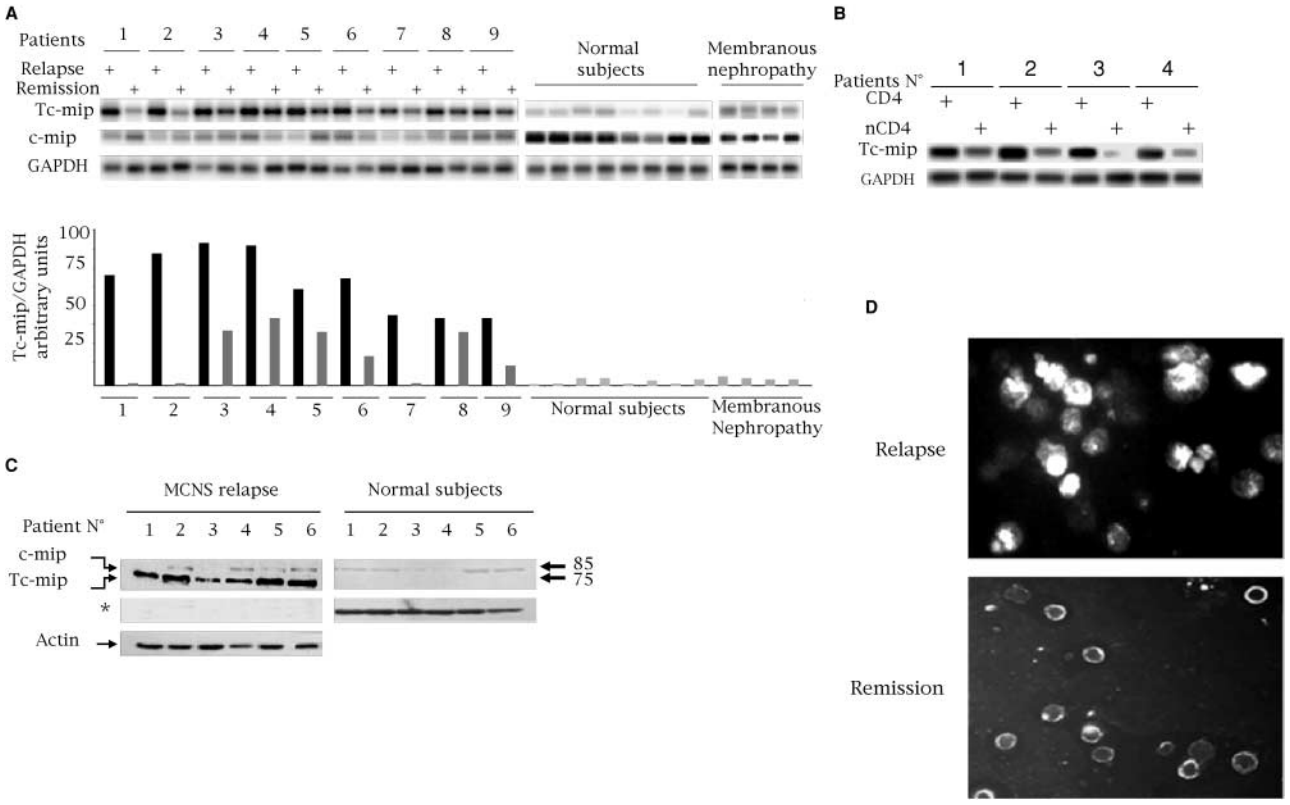

Figure 4. Specific induction of Tc-mip in MCNS relapse. (A) RT-PCR analyses of Tc-mip mRNA expression. Nine patients with MCNS were studied in relapse and in remission as well as eight normal subjects and four nephrotic patients with membranous nephropathy. The expression of GAPDH was monitored in parallel, in order to control the initial amount of mRNA. The bottom panel shows quantification of the PCR bands in panel A determined by using the Image Quant V 1.11 analysis software, after normalization against the corresponding GAPDH bands; (B) induction of Tc-mip was higher in the $CD4^+$ T cell subset. Total RNA from $CD4^+$, non-$CD4^+$ (nCD4) subsets was purified from PBMC of patients with MCNS relapse, as described under Materials and Methods and analyzed for Tc-mip expression by RT-PCR. (C) Western blot analyses using anti-Tc-mip/c-mip antibody. Total protein extracts (50 μg) of PBMC from six patients in relapse and six normal subjects were tested for Tc-mip/c-mip expression; the specificity of staining was demonstrated by the loss of signal upon preincubation of antibody with immunogenic peptide (asterisk). The blots were stripped and re-probed with anti-actin antibodies; (D) immunostaining of PBMC of a pediatric patient with MCNS (4-yr-old, first relapse and remission). PBMCs were purified, methanol fixed and incubated with anti-Tc-mip/c-mip antibody, as described under Materials and Methods (×40, magnification).

disease. We purified by immunomagnetic selection, the $CD4^+$ and non-$CD4^+$ T cells species from PBMC of patients with relapse. The highest level of Tc-mip mRNA was observed in the $CD4^+$ T cell subset (Fig. 4 B).

As we could not detect Tc-mip protein in normal PBMC cells, we determined whether it was induced during MCNS disease. We immunoblotted total protein extracts from PBMC of six patients in relapse. We detected a major band of 75 kD specific of Tc-mip in patients with MCNS relapse whereas the signal was undetectable in normal subjects (Fig. 4 C). In contrast, the expected 85 kD band related to c-mip was weakly detectable in MCNS patients as well as in normal subjects.

Cellular distribution of Tc-mip was analyzed in PBMCs from patients with MCNS. In relapse, we detected Tc-mip in nuclear and cytoplasmic compartments, whereas its expression was restricted to the cytoplasm in remission (Fig. 4 D). In those samples, 20% of the PBMCs were Tc-mip positive whereas no cellular staining was visualized in PBMC from normal controls in accordance with the Western blotting results (unpublished data).

4-Tc-mip Induces c-maf Nuclear Expression in Jurkat Cells. Recently, we have shown that c-maf was strongly induced in MCNS and shuttled between nuclear and cytoplasmic compartments during the relapse and the remission phases, respectively (unpublished data). As Tc-mip and c-maf are mainly expressed in $CD4^+$T cells, we examined whether Tc-mip could influence c-maf expression. We transfected Jurkat cells, which do not express either endogenous c-mip or Tc-mip, with c-mip or Tc-mip expression vectors. Transfection with Tc-mip induced c-maf expression at the mRNA and protein level (Fig. 5 A, top panel), whereas c-mip promoted a lower but significative induction of c-maf relative to controls, the efficiency of transfection being similar (Fig. 5 A, bottom panel). Importantly, the expression pattern of the c-maf protein was radically different: in cells overexpressing Tc-mip, c-maf was primarily detected in nuclei (Fig. 5 B, top panel), whereas its expression was much lower and mostly restricted to periphery of the cell in c-mip transfected cells (Fig. 5 B, middle panel). The induction of c-maf and its nuclear localization in transfected cells did not indicate that c-maf is transcriptionally active. To

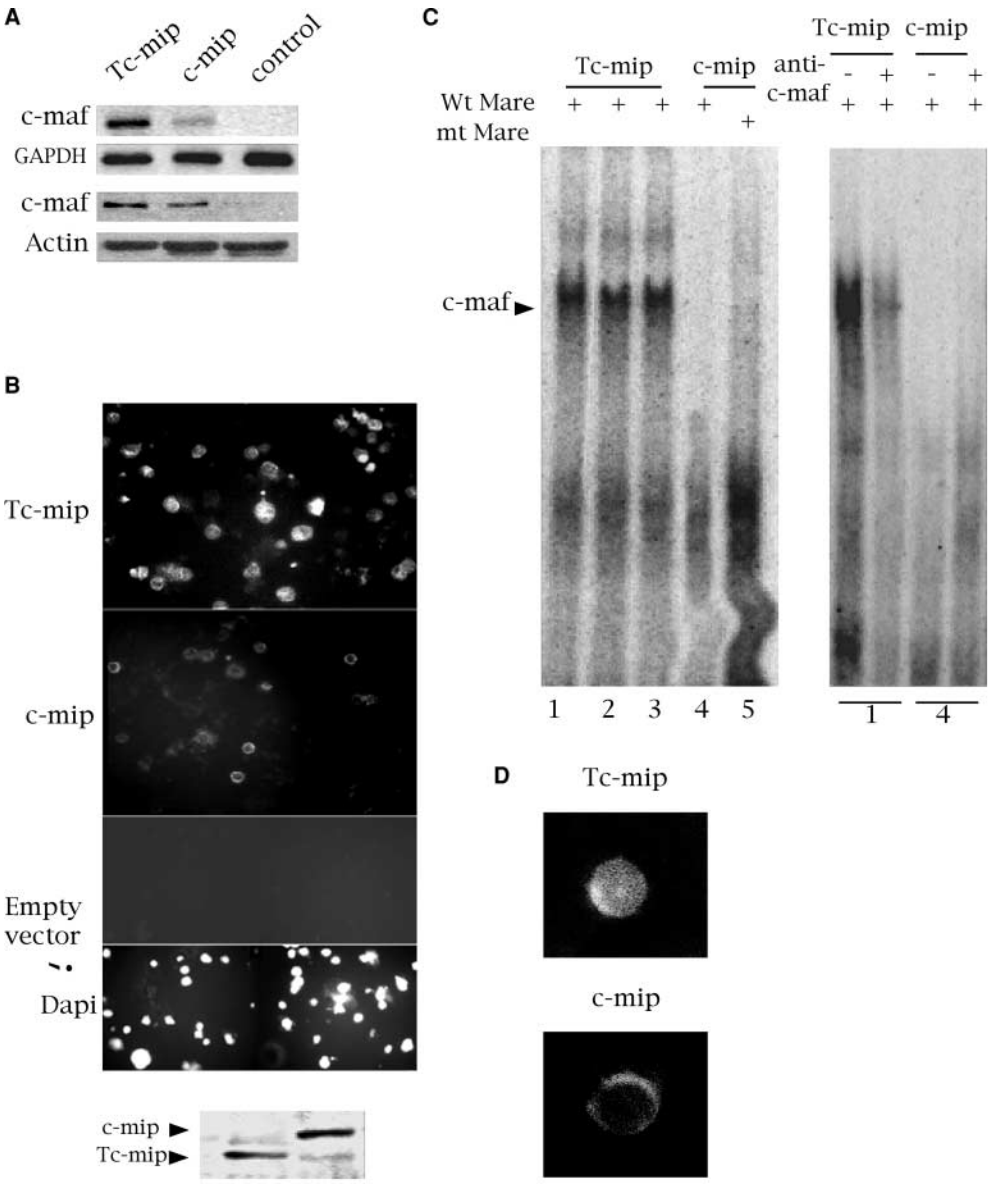

Figure 5. Tc-Mip induces c-maf nuclear expression. (A) RT-PCR and Western blot analyses for c-maf expression in Jurkat cells transfected with either Tc-mip, c-mip, or empty vector, as described under Materials and Methods. (B) Top panel, immunofluorescence detection of c-maf in transfected Jurkat cells. The data are representative of six separate experiments. Bottom panel, Western blotting of cytosolic extracts from transfected cells, using anti-c-mip/Tc-mip antibody. (C) c-maf DNA binding activity. EMSA analyses of nuclear extracts (15 μg) from Tc-mip- (lane 1–3) and c-mip- (Lane 4) transfected Jurkat cells. The specificity of the band shift (black arrow) was demonstrated by the loss of DNA binding when the mutant Mare (mt Mare) was substituted to wild-type Mare sequence (line 5) and in the presence of anti-c-maf antibody (right of the Fig. 3 C). (D) Confocal imaging of Tc-mip and c-mip after transient transfection in Jurkat cells with respective expression vectors. This result is representative of several cells in four independent experiments.

address this question, we analyzed by EMSA experiments, c-maf–dependent DNA binding activity in nuclear extracts of Tc-mip–transfected Jurkat cells. We only detected a slow migrating complex binding to MARE probe in Tc-mip transfected Jurkat cells whereas no complex was visible in c-mip transfected cells This band shift was specific since it was not formed in the presence of mutant MARE oligonucleotide (Fig. 5 C). Moreover, the specificity of DNA binding was demonstrated by the loss of the band shift in the presence of anti-c-maf polyclonal antibody, as reported by others (12), suggesting that antibody competes c-maf for binding to the Mare sequence. These results suggest that Tc-mip induced a functional c-maf in Jurkat T cells.

Confocal fluorescence microscope analysis revealed that the c-mip protein was restricted to peripheral area of transfected cells, whereas Tc-mip was diffusely expressed, particularly in nuclear compartment (Fig. 5 D). Thus, Tc-mip and c-maf display a similar distribution in nuclei and cytoplasmic compartments but we were not able to coimmunoprecipitate both proteins. Altogether, these results suggest that truncation of the PH domain affects the cellular distribution of the Tc-mip protein, which might regulate the induction and the cellular compartmentalization of c-maf.

5-Overexpression of Tc-mip Results in Differential Effects on IL-4 and IFN-γ Production. As c-maf is a transactivator of the IL-4 gene (5, 13), the identification of Tc-mip as an upstream inducer of c-maf led us to determine whether Tc-mip could be able to activate the IL-4 gene. To that end, we analyzed the effects of a transient transfection of Tc-mip on IL-4 promoter activity in Jurkat cells. In unstimulated-transfected Jurkat cells, no significant variation of the IL-4–dependent CAT activity was detected after transfection with Tc-mip (Fig. 6 A). When transfected Jurkat T cells were stimulated with anti-CD3 antibody, Tc-mip and c-mip

induced a fourfold and twofold increase of Il-4 promoter activity relative to control, respectively (Fig. 6 B).

As Tc-mip induces the expression of c-maf, we supposed that these cells acquired a Th2 phenotype. To test this hypothesis we analyzed in parallel IFN-γ and IL-4 mRNA expression, two reliable markers of Th1 and Th2 profile, respectively. In unstimulated transfected T cells, Tc-mip, and c-mip did not alter significantly the IL-4 and IFN-γ mRNA expressions, relative to controls (Fig. 5 B). Upon stimulation with anti-CD3 antibody, IL-4 mRNA expression was considerably increased, whereas IFN-γ mRNA expression was sharply reduced in Tc-mip-overexpressing cells (Fig. 6 B). By contrast, c-mip did not reduce IFN-γ mRNA, whereas the IL-4 mRNA was slightly increased relative to Tc-mip–transfected cells. This result suggests that signals emanating from TCR engagement provided additional requirement for c-maf-mediated Th2 pathway in which Tc-mip but not c-mip might play a potent role.

6-Overexpression of Tc-mip Is Associated with T Cell Activation and Cellular Redistribution of Cytoskeleton-associated L-plastin. Our precedent results support the role of TCR activation in c-maf signaling pathway, as it has been reported previously (14), but upstream proteins involved in c-maf induction remain unidentified. As Tc-mip behaves as a proximal inducer of c-maf expression, we wished to determine whether Tc-mip could mimic TCR signaling. To test this hypothesis, we analyzed the distribution of L-plastin, a cytoskeleton protein that is up-regulated in active MCNS disease (3). L-plastin is an actin bundling protein that plays an important role in microspike and filopodia formation. It is activated after costimulation via an accessory receptor such as CD2 and CD28. Activation of l-plastin is required for full T cell activation and stabilization of immunological synapse by clustering receptors and bundling actin filaments at the T cell/Ag presenting cell contact area (15, 16). We transfected Jurkat T cells with Tc-mip and c-mip expression vectors and 16 h later, cells were recovered from medium alone, fixed and incubated with L-plastin monoclonal antibody. T cells transfected with Tc-mip induced a cell clustering with distribution changes of L-plastin that was relocalized in cytoplasmic zones facing the cell contact (Fig. 7 A, top panel), whereas c-mip, transfected with a similar efficiency (Fig. 7 A, bottom panel), exhibited an homogeneously distribution of L-plastin in the cytoplasm area. We conclude that overexpression of Tc-mip promotes the cytoskeleton rearrangement. To support this data, we analyze the status of Src protein tyrosine kinase, a proximal signaling protein that is recruited early in T cell activation. Protein extracts from Tc-mip, but not c-mip, overexpressed T cells displayed a strong signal with anti-phospho-Src (Tyr 416; Fig. 7 B). To assess whether this effect involved the PI3-kinase pathway, we treated Tc-mip–transfected cells with wortmanin, a PI3-k inhibitor. We found that cell clustering as well as the relocalization of L-plastin were not affected by wortmanin treatment (Fig. 7 C). These results suggest that Tc-mip induced T cell activation and cytoskeleton reorganization by a mechanism independent of PI3 kinase activity.

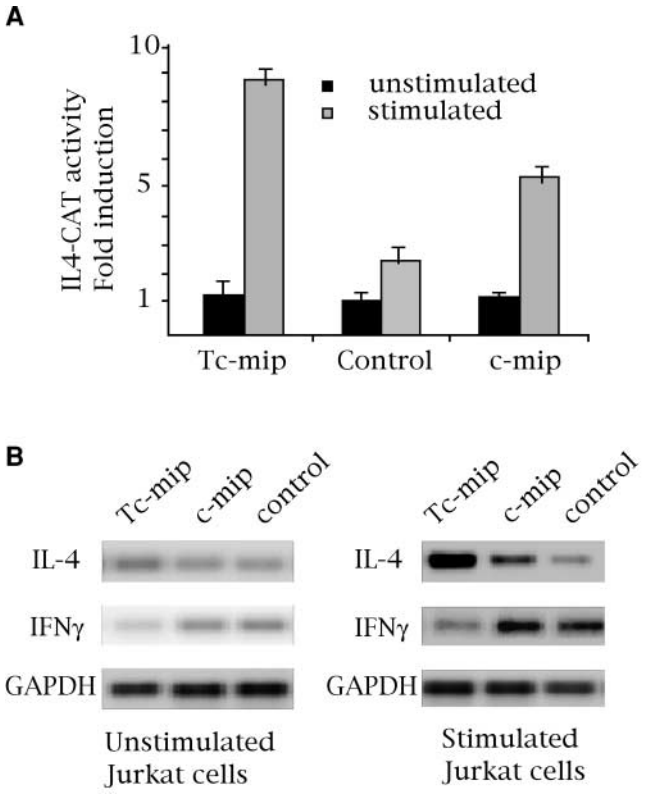

Figure 6. Tc-Mip induces the transactivation of the IL-4 gene and inhibits the IFN-γ expression in stimulated Jurkat T cells. (A) Jurkat T cells were transiently cotransfected with the IL-4/CAT reporter vector and either Tc-mip, c-mip, or empty vector. Cells were then incubated with medium alone or with anti-OkT3 monoclonal antibody as described under Materials and Methods. Data are expressed as fold induction of intracellular CAT and represent the mean results of four independent experiments. (B) RT-PCR of total RNA extracted using IL-4 and INF-γ specific primers (Table I). The expression of GAPDH was monitored in parallel.

Discussion

We isolated from T lymphocyte of patients with MCNS a new signaling protein, Tc-mip, which induce T cell activation, cytoskeleton redistribution, and c-maf transcription factor. Tc-mip corresponds to a truncated form of the protein c-mip, both proteins being produced by a single gene through two alternative transcripts. The main difference between these proteins is a deletion in Tc-mip of the NH_2-terminal part of a PH domain, which is fully functional in c-mip. These two proteins display striking differences in cellular expression and distribution, as well as in functional characteristics.

The selective expression of Tc-mip in thymus and fetal liver contrasts with its modest detection in normal peripheral T cells. Interestingly, the c-mip transcript is mostly expressed in mature PBMC and the fetal liver signal detected with the common probe presumably reflects the contribution of Tc-mip. These data suggest that Tc-mip is expressed by normal lymphocyte precursors but this expression shuts down during maturation and differentiation processes occurring in thymus and fetal liver.

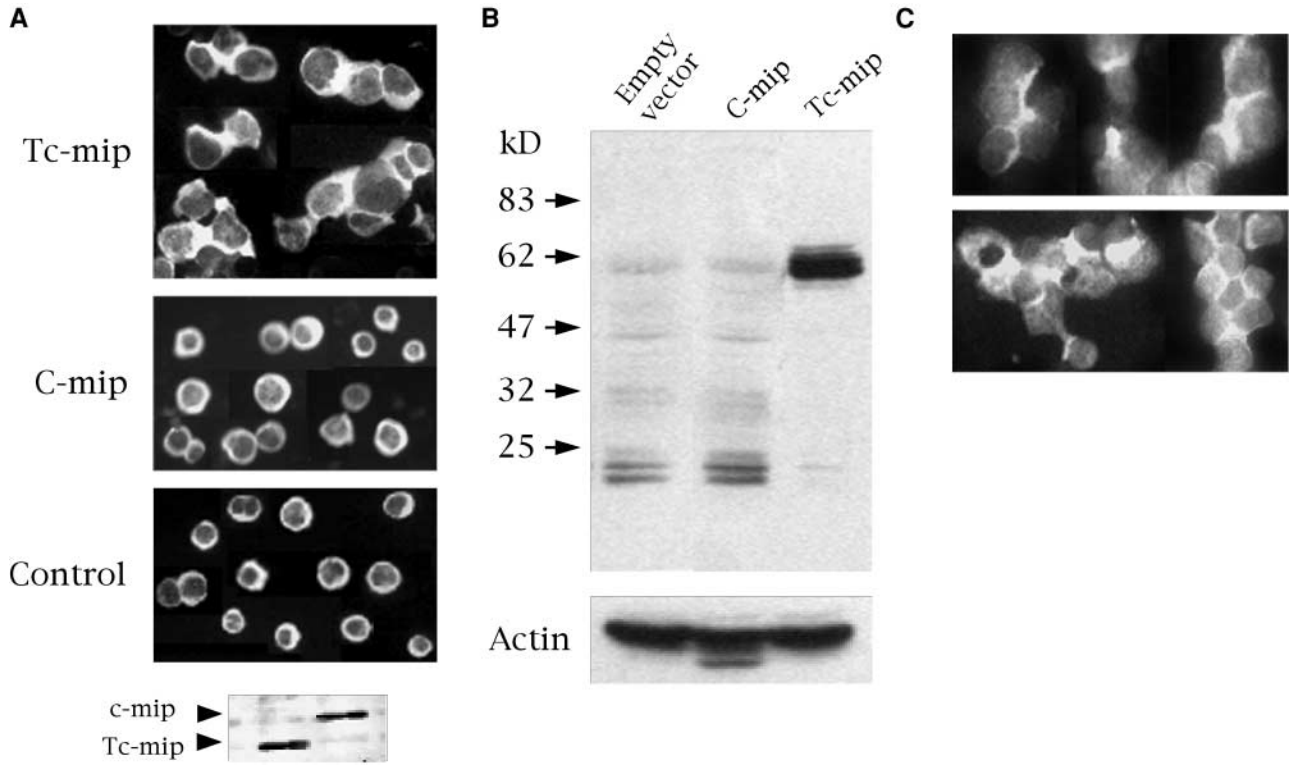

Figure 7. Tc-mip induces T cell activation and cytoskeleton reorganization by a PI3 kinase-independent mechanism. (A) Top panel, immunofluorescence detection of L-plastin in unstimulated Jurkat cells transfected with either Tc-mip, c-mip or empty vector. The data are representative of three independent experiments. Bottom panel, Western blotting of cytosolic extracts from transfected cells, using anti–c-mip/Tc-mip antibody. (B) Western blot analyses of protein extracts (50 μg) from empty vector- (lane 1), c-mip- (lane 2), and Tc-mip- (lane 3) transfected Jurkat cells, using anti-phospho Src antibody (Tyr 416). (C) Pharmacologic inhibitors of PI3-K did not inhibit redistribution of cytoskeleton-associated L-plastin. Jurkat T cells transfected with Tc-mip and wortmanin (100 nM) was added to medium culture, six hours following transfection and left overnight. This result was reproduced in three independent experiments.

We showed that Tc-mip is specifically expressed in $CD4^+$ T cells in patients with MCNS and plays a driving role in c-maf–mediated Th2 signaling pathway. Such findings extend our previous data regarding the specific recruitment of c-maf in MCNS (unpublished data). Conversely, c-mip is mainly detectable in PBMCs of normal subjects and exerts slight effects on c-maf expression that is restricted to cytoplasmic compartment.

Under unstimulated conditions, c-mip and Tc-mip proteins were weakly or not detected despite the basal expression of their respective transcripts, as reported for other genes expressed in immune cells (17). Upon stimulation by pharmacological agents that increase $(Ca^{2+})i$ such as PMA/ionomycin, we observed a dramatic fall in Tc-mip and c-mip mRNA levels. By contrast, these agents did not apparently influence the expression of c-maf in similar culture conditions of normal cells (18, 19), suggesting that regulation of c-maf in physiological conditions is independent of Tc-mip. It is likely that PMA/ionomycin activate PKC, which exerts a negative regulatory role on Tc-mip/c-mip signaling pathway as recently demonstrated for the PI3k/AKT pathway (20). On the other hand, the stimulation by PMA/ionomycin failed to induce a down-regulation of Tc-mip in MCNS T cells, suggesting that alteration of this signaling pathway contribute to T cell dysfunction in this disease.

Under Th skewing conditions, Tc-mip is preferentially recruited in Th2 but not in Th1 cells. In addition c-maf is induced in Tc-mip–transfected T cells and there is an increase of IL-4 promoter activity. The IL-4 induction in Tc-mip-, and to a lesser extent, in c-mip–overexpressing T cells, was observed only after TCR engagement after stimulation by the anti-CD3 antibody. These data suggest that additional signals, stemming from TCR, are required for the IL-4 induction by c-maf, as reported previously (14).

Intracellular molecules, known as cytosolic adaptor proteins (CAPs) and lacking intrinsic enzymatic or transcriptional activity, contain modular domains which serve to recruit proteins to couple proximal events initiated by TCR ligation with more distal signaling pathways. Tc-mip/c-mip might fall into this CAP family as the first protein identified so far, linking TCR proximal signaling to c-maf transcription factor.

By comparison, NFATc-mediated Th2 signaling uses apparently distinct pathway, involving the activation of lck and the phosphactivation of the PH domain-containing protein Itk/Emt, a T cell associated Tec kinase (21). As matter of fact, Th2 development fails in lck-deficient T cells (22). Nuclear localization of NFATc is sharply reduced in itk-deficient T cells upon stimulation by anti-TCR/CD28 (23). However, activation of PKC with cal-

cium ionophore restores NFATc-mediated IL-4 expression. Importantly, Itk does not exhibit any influence on Th1 cytokines (24). These data fundamentally differentiate Itk/Emt from Tc-mip by at least two aspects: (a) Tc-mip bypasses the requirement for calcium signaling; (b) Tc-mip induces c-maf and represses the IFN-γ expression. Nevertheless, whether the down-regulation of IFN-γ is a direct effect of Tc-mip or a consequence of the induction of c-maf remains to be clarified.

PH domain-containing proteins are members of a large family involved in cellular signaling (25, 26, 27). The consensus sequence of the PH domains is weak but they share similar core β sandwich structure (28). The PH domains comprise ~100 amino acids, likely to recruit molecules to the membrane by specific interactions with phosphoinositide lipids (28). These interactions might contribute to the attachment of the plasma membrane to the cytoskeleton through lipid–protein and protein–protein interactions leading to a regulation of the cytoskeleton organization (29). We showed here that Tc-mip partially mimics TCR signaling and induced T cell clustering and refolding of the cytoskeleton protein L-plastin, independently of PI3 kinase pathway.

The truncation of the PH domain of Tc-mip is associated with a nuclear location of the protein. This suggests that the PH domain of c-mip acts cooperatively with membrane phosphoinositides to cluster c-mip at and near the plasma membrane. The nuclear translocation of Tc-mip might result from the alteration of the β sheet structure present in the phosphoinositide-binding module of the PH domain of c-mip, as previously shown for other PH domain-containing proteins (29–31).

The inhibitory role of the PH domain on cellular signaling has been documented for other proteins. The PH domain of the guanine nucleotide exchange factors (GEFs) Vav-1 acts as a negative, intramolecular regulator of Dbl homology (DH) domain function. Mutations in the PH domain alters the phosphoinositide binding of Vav-1 and induces an increase in GEF activity (32). In addition, a Vav variant, lacking the PH domain, induces JNK activation leading to cytoskeletal reorganization (33). As other examples, deletion of the PH domain of the protein kinase D (PKD) or point mutation in PH domain from Bruton's tyrosine kinase (BTK), increased kinase activities (34, 35).

Alterations of the PH domain have also been associated with several human disease: (a) a point mutation in the PH domain of Btk is associated with X-linked agammaglobulinaemia (36); (b) alterations in the DH-PH domain of proteins such as Ost, Lfc, Dbs, or the FGD-1 gene are implicated in human oncogenesis and developmental disorders (37–40).

Inasmuch that c-mip constitutes the normal protein isoform in T lymphocytes of normal subjects, the occurrence of Tc-mip at high level in MCNS patients allows important regulatory changes in the putative signaling pathway. Truncation of the PH domain might suppress the anchorage of Tc-mip to membrane-phosphoinositides, precluding proximal interactions, which could be negatively regulate T-cmip. Nevertheless, Tc-mip conserves other domains allowing its interactions with downstream signaling molecules. After TCR stimulation, Tc-mip up-regulates the activation cascades that ultimately induce c-maf and affect its cytokine repertoire.

In conclusion, we identified in this study a new protein, Tc-mip, which is specifically recruited during MCNS. We showed that Tc-mip links TCR-mediated signal to the activation of c-maf Th2 specific factor. The presence of a truncated PH domain allows a nuclear translocation of the protein, which promote a high c-maf expression level in MCNS patients. It also gives additional evidence regarding the inhibitory function of the PH domain on protein activity and its critical role on protein localization. Finally, it gives strong support to the potential involvement of unknown alternative species of transcripts in human diseases.

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