Cloning and Characterization of N4WBP5A, an Inducible, Cyclosporine-sensitive, Nedd4-binding Protein in Human T Lymphocytes*

We have cloned and characterized a human cDNA, designated N4WBP5A, that belongs to the family of Nedd4-binding proteins. We originally identified N4WBP5A as an unknown expressed sequence tag (AA770150) represented in a cDNA microarray analysis that was up-regulated upon activation of T cells and inhibited by cell treatment with the calcineurin phosphatase inhibitors, cyclosporine (CsA) and tacrolimus (FK506). The predicted N4WBP5A amino acid sequence of 242 amino acid residues reveals an open reading frame of 729 nucleotides with a corresponding molecular mass of 27.1 kDa. Detection of N4WBP5A mRNA by reverse transcription-PCR was consistent with the induction of N4WBP5A following mitogenic stimulation of T lymphocytes and inhibition by CsA. Immunoblot analysis revealed endogenous N4WBP5A protein to be up-regulated following T cell activation and inhibited by CsA. This regulation of N4WBP5A mRNA by reverse transcription-PCR was consistent with the induction of N4WBP5A following mitogenic stimulation of T lymphocytes and inhibition by CsA. Immunoblot analysis revealed endogenous N4WBP5A protein to be up-regulated following T cell activation and inhibited by CsA. This regulation of N4WBP5A mRNA expression differed from that of its homologue (51% identical; 65% similar) N4WBP5. Like N4WBP5, however, expression of epitope-tagged N4WBP5A indicated that the protein is localized predominantly to the Golgi network. Here we show by co-precipitation experiments that N4WBP5A interacts with the WW domains of Nedd4, an E3 ubiquitin ligase. Taken together, our data suggest that N4WBP5A may play a regulatory role in modulating Nedd4 activity at the level of the Golgi apparatus in T lymphocytes.

Ubiquitination is a post-translational modification of proteins that involves the covalent binding of a 76-amino acid polypeptide, ubiquitin, to a target protein (1–3). This multistep process is mediated by the proteins, E1, E2, and E3,† that flag protein substrates destined for rapid proteasome-driven degrada-

tion. The ubiquitin-activating enzyme, E1, is responsible for ATP-dependent ubiquitin activation. Once activated, ubiquitin is transferred to the downstream carrier protein, E2, and then to the isopeptide ligase, E3, that mediates the transfer to a substrate protein (3). Ubiquitination is involved in the down-regulation of membrane receptors, transporters, and channels as well as in cell cycle control (4). Within the immune system, ubiquitination is involved in transcriptional and translational activation, protein kinase activation, and apoptosis (1). Although the enzymes involved in ubiquitination have been extensively studied, the factors and signals that regulate their activities remain less well understood. Resolving this regulation is important given the evidence that irregular ubiquitina-

tion can give rise to cellular dysregulation (5–7).

To identify regulatory signals that may control ubiquitination, the enzyme-substrate recognition step, mediated by E3, has received more attention in recent years. A growing family of E3 ubiquitin ligases, termed Nedd4, share an N-terminal Ca2+-dependent lipid protein binding (C2/CaLB) domain (8), two to four WW protein-protein interaction domains (9), and a C-terminal Hect (homology to E6-associated protein at the carboxyl terminus) domain. Although the C2/CaLB domain is responsible for membrane localization in response to calcium signals, the conserved Hect domain represents the catalytic region responsible for ubiquitin ligase activity. Nedd4, initially identified to be developmentally down-regulated in neuronal precursor cells (10), targets downstream substrates for ubiquitination and subsequent degradation. Nedd4 is evolutionar-

ily well conserved: orthologues in yeast, mouse, rat, and humans have been identified (11). At present, the best characterized Nedd4 target is the amiloride-sensitive epithelial sodium channel (ENaC) (12–14). ENaC is involved in renal sodium transport and in the regulation of blood pressure. Deletion of the WW-binding domains of ENaC leads to hyperac-

tivation of the channel, a characteristic of patients with Liddle's syndrome.

The Nedd4 WW domains are small regions comprising 38–40 conserved amino acid residues that mediate protein-protein interactions (15–17) by binding target proteins containing the consensus sequence, X(PPXY) (PY motif) (16, 17). Using mouse embryonic cDNA expression libraries, Jolliffe and co-workers (18) identified eight PY motif-containing murine pro-

teins capable of binding Nedd4 in vitro. One of the identified proteins, N4WBP5, was later shown to be Golgi-localized and capable of binding Nedd4 via an interaction of the PY motifs (of N4WBP5) with the Nedd4 WW domains (19).

We here report the cloning and characterization of N4WBP5A, a cDNA encoding a novel member of the growing family of Nedd4-binding proteins, isolated from activated human peripheral blood T lymphocytes. The 729 nucleotide open

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† The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; PBLS, peripheral blood T lymphocytes; FMA, phorbol 12-myristate 13-acetate; N4WBP, Nedd4 WW-domain-binding protein; ENaC, epithelial sodium channel; EST, expressed sequence tag; CsA, cyclosporine; RT, reverse transcription; PBS, phosphate-buffered saline; mAb, monoclonal antibody; GFP, green fluorescent protein; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; PVDF, polyvinylidene difluoride; DAPI, 4',6-diamidino-2-phenylindole; ER, endoplasmic reticulum.

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Fig. 1. Amino acid and nucleotide sequence analysis of N4WBP5A. A, amino acid alignment of N4WBP5A and N4WBP5 cDNA is shown (GenBank™ accession number is XP_041162 for N4WBP5A and NP_085048 for N4WBP5). Identical (red), similar (green), and different (black) amino acid residues are indicated. B, exon/intron composition of N4WBP5A on chromosome 13 (upper panel, Gene Finder software, Sanger.
MATERIALS AND METHODS

Cells and Stimulations—Human peripheral blood lymphocytes (PBLs) were obtained from healthy human donors, isolated by apheresis, the passage of flow elutriation and centrifugation through Ficoll-Hypaque, and washed with 1× phosphate-buffered saline (PBS). PBLs were resuspended in RPMI 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal calf serum (Hyclone, UT), 2 mM l-glutamine, 10 mM Hepes, pH 7.2, 100 units/ml penicillin, 100 μg/ml streptomycin (MediaTech), and 50 μg/ml 2-mercaptoethanol (Bio-Rad, Hercules, CA), termed 10% FBS (RF11640), and incubated at 37 °C, 5% CO₂ in air. After overnight incubation, cells were stimulated with 10 ng/ml phorbol 12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA), and/or 1 μg/ml anti-CD3 mAb OKT3 (American Type Culture Collection, Rockville, MD), SH3 binding domains (red), myristoylation (blue), and (iii) 72 °C for 10 min. The resulting N4WBP5A cDNA PCR product (934 nucleotides) was subsequently cloned using BglII/SalI restriction enzymes into the corresponding multiple cloning site of pEGFP-C3 (Clontech), hereinafter termed GFP-N4WBP5A. N4WBP5A was PCR-amplified from HA-N4WBP5A using the following primers: N4WBP5F, 5'-AGG GAT CTA CCA TGG ATC ACC ACC CCG GG-3'; N4WBP5R, 5'-GCT CTA GAA TGT CGG CAG CAG CAA AAA CCA CCC TGC CAT TCA ATG TCT-3'. PCR was performed using the following conditions: 94 °C for 45 s; 30 cycles of (i) 94 °C for 45 s, (ii) 72 °C for 45 s, and (iii) 72 °C for 2 min; and 1 cycle of 72 °C for 10 min. The resulting N4WBP5A cDNA PCR product (934 nucleotides) was labeled with [α-32P]dCTP (3000 Ci/mmol, 10 mCi/ml) using a random primer labeling procedure (see “Materials and Methods”) and used to probe a multiple tissue Northern blot membrane (Clontech) according to the manufacturer’s protocol.

CA) according the manufacturer’s protocol and quantitated using the RiboGreen™ RNA quantitation kit (Molecular Probes, Eugene, OR). A reverse transcription reaction was carried out using 50 ng of sample mRNA and an oligo(dT)12–18 primer (Invitrogen). N4WBP5A cDNA was isolated from the resulting cDNA by PCR using PfuTurbo® polymerase (Stratagene, La Jolla, CA) and the following primers: N4WBP5F, 5'-ACG CTT CGA CGC GAG AAG ACT CCC TCT CTC-3'; N4WBP5R, 5'-ATA AGA ATG CCG CAG CAA AAA CCA CCC TGC CAT TCA ATG TCT-3'. RT-PCR analysis showed N4WBP5A mRNA to be expressed in activated but not resting T cells. Induction of both N4WBP5A mRNA and protein was found to be sensitive to inhibition by Ca²⁺ and FK506, but not rapamycin, and, hence, regulated by calcineurin. N4WBP5A was found to localize predominantly to the Golgi network and to interact with Nedd4 via binding to the Nedd4 WW-1, WW-2, and WW-3 domains. While the manuscript for this report was in review, the same protein was reported to have been cloned from epithelial sources and found to bind Nedd4 (21). Our findings reveal that the novel member of the Nedd4 WW domain-binding protein family is induced in activated T cells; a role for Nedd4 and its binding partners, and for Nedd4-dependent ubiquitination pathways in human T lymphocytes has not been previously appreciated.

Isolation of N4WBP5A—Human PBLs were stimulated with plate-bound anti-CD3 mAb plus PMA for 4 h. Total RNA was prepared using Trizol™ (Invitrogen) and quantitated using A260. Poly(A) RNA (mRNA) was isolated using the oligotex mRNA kit (Qiagen, Valencia, CA) according the manufacturer’s protocol and quantitated using the RiboGreen™ RNA quantitation kit (Molecular Probes, Eugene, OR). A reverse transcription reaction was carried out using 50 ng of sample mRNA and an oligo(dT)12–18 primer (Invitrogen). N4WBP5A cDNA was isolated from the resulting cDNA by PCR using PfuTurbo® polymerase (Stratagene, La Jolla, CA) and the following primers: N4WBP5F, 5'-ACG CTT CGA CGC GAG AAG ACT CCC TCT CTC-3'; N4WBP5R, 5'-ATA AGA ATG CCG CAG CAA AAA CCA CCC TGC CAT TCA ATG TCT-3'. N4WBP5A was subsequently cloned using BglII/SalI restriction enzymes into the corresponding multiple cloning site of pEGFP-C3 (Clontech), hereinafter termed GFP-N4WBP5A. N4WBP5A was PCR-amplified from HA-N4WBP5A using the following primers: N4WBP5F, 5'-AGG GAT CTA CCA TGG ATC ACC ACC CCG GG-3'; N4WBP5R, 5'-GCT CTA GAA TGT CGG CAG CAG CAA AAA CCA CCC TGC CAT TCA ATG TCT-3'. PCR was performed using the following conditions: 94 °C for 45 s; 30 cycles of (i) 94 °C for 45 s, (ii) 72 °C for 45 s, and (iii) 72 °C for 2 min; and 1 cycle of 72 °C for 10 min. The resulting N4WBP5A cDNA PCR product (934 nucleotides) was labeled with [α-32P]dCTP (3000 Ci/mmol, 10 mCi/ml) using a random primer labeling procedure (see “Materials and Methods”) and used to probe a multiple tissue Northern blot membrane (Clontech) according to the manufacturer’s protocol.

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RT-PCR—Total RNA was prepared from human PBLs using Trizol™ and quantitated using A260. N4WBP5A was PCR-amplified from whole human lymphocytes using the following oligonucleotide purification cartridge-purified primers (BioServe Biotechnologies, Laurel, MD): N4WBP5F, 5'-TCA GCA CCA GCT GAA ACA AA-3'; N4WBP5R, 5'-ATC TGG CAC ACC ACC TCA ATC AAT GAG CTG CG-3'; and β-actin-R, 5'-CGT CAT ACT GCT TGC TGA TCG CCG CG-3'.

Institute, UK is highlighted in the schematic of the 7 exons (black). introns (white), and 5' untranslated region (gray). Putative transcription factor binding sites in the promoter (lower panel, TRANSFAC (22)) are shown. C, a schematic representation of the predicted protein domains of N4WBP5A is shown. Panel, an integrated search of PROSITE, Pfam, PRINTS, and other family and domain databases (23). Putative SH2 binding sites (light purple), SH3 binding domains (red), proline-rich WW binding domains (dark purple), transmembrane regions (green), myristoylation site (blue), and predicted Ser/Thr phosphorylation sites (orange) are indicated.
ACA TCT GC-3’. RT-PCR was performed using the following conditions: 50 °C for 30 min; 95 °C for 15 min; 30 cycles of (i) 94 °C for 1 min, (ii) 55 °C for 1 min, and (iii) 72 °C for 1 min; and 72 °C for 10 min. Samples were analyzed by gel electrophoresis, and bands were revealed by staining gels with ethidium bromide. Bands were quantitated by phosphorimaging analysis using ImageQuaNT software, and mRNA levels were normalized to β-actin mRNA levels as indicated.

**Antibody Production**—Two rabbits were inoculated with 0.5 mg of a KHL-conjugated peptide (QEECPPRDFFSDADQ, Covance Immunological Service, Denver, PA) specific for N4WBP5A. Rabbits were boosted four times with peptide, 3 weeks apart, using 0.25 mg/boost in Freund’s Incomplete Adjuvant. Sera were collected and tested against the antigenic peptide using enzyme-linked immunosorbent assay (data not shown). Collected sera showing the most robust and specific reactivity for antigenic peptide (clone 1442) were subsequently used for Western blot protein analysis.

**Precipitations and Characterization of Nedd4 Association**—GFP-N4WBP5A or pEGFP-C3 (term GFP) were transiently transfected into COS-7 and HeLa cells using the CalPhos mammalian transfection kit (Clontech) according to the manufacturer’s instructions. Following transient transfection, cells rested for 24 h at 37 °C prior to harvesting by centrifugation at 466 × g for 5 min at 4 °C. Cells were washed once with cold DMEM (without additives), and 105 cells were resuspended at a concentration of 107 cells/100 μl in DMEM prior to centrifugation at 9,000 × g for 5 min at 4 °C.

**Immunofluorescence**—COS-7 cells (5 × 104 cells/coverslip) were plated on glass coverslips and transiently transfected with GFP-N4WBP5A or a control GFP construct. Following transfection (24 h), cells were analyzed by immunofluorescence. Briefly, cells were fixed in 3.7% formaldehyde for 20 min at room temperature. Coverslips were washed three times using 1× PBS and incubated with 0.05% Triton X-100 in PBS for 2.5 min at room temperature. Coverslips were washed three times using 0.2% gelatin in PBS and incubated with anti-GM130 (BD Transduction Laboratories) in antibody dilution solution (1× PBS, 0.2% bovine serum albumin, 0.1% sodium azide) at room temperature for 30 min to stain cells for the Golgi complex. Alternatively, the nucleus was detected using either Hoechst (Sigma-Aldrich, St. Louis, MO) or DAPI (Sigma-Aldrich), endoplasmic reticulum detected using ER-Tracker Blue-White DPX dye (Molecular Probes) and mitochondria detected using MitoTracker Red-CMXRos dye (Molecular Probes). Coverslips were washed three times with 0.2% gelatin in PBS, three times with 1× PBS, mounted onto glass slides (using a Prolong Antifade kit, Molecular Probes), and visualized using confocal fluorescence microscopy (Zeiss LSM510; a 100×/1.4 numerical aperture oil lens was used for all samples except for nuclear staining: 63×/1.2 water lens). Endogenous N4WBP5A was detected in COS-7 cells and human PBLs by immunofluorescence and confocal microscopy using anti-antiserum (clone 1442) followed by either fluorescein- or phycoerythrin-conjugated anti-rabbit secondary antibody (Molecular Probes). For N4WBP5A and Nedd4 co-localization studies, 293T cells were co-transfected with GFP-N4WBP5A and GFP-Nedd4 constructs using the CalPhos mammalian transfection protocol as outlined above. Expression of these targets was visualized using confocal microscopy as previously outlined. Endogenous N4WBP5A was detected in 293T cells using anti-antiserum...
(clone 1442) and a fluorescein-conjugated anti-rabbit secondary antibody (Molecular Probes). Endogenous Nedd4 was detected in 293T cells using a mouse anti-Nedd4 monoclonal antibody (BD Pharmingen) and a phycoerythrin-conjugated anti-mouse secondary antibody (Molecular Probes).

RESULTS

N4WBP5A: a Novel Nedd4 WW Domain-binding Protein Family Member—We originally identified N4WBP5A as an unknown EST represented in a cDNA microarray analysis that was induced following T cell activation and inhibited by the immunosuppressive agents, cyclosporine (CsA) and tacrolimus (FK506) (20). A GenBank™ nucleotide search (BLASTn) using this EST (AA770150) of the non-redundant data base (GenBank™/EMBL/DDBJ/PDB) of sequences revealed a cDNA sequence containing the EST of interest, encoding a protein of no known function (KIAA1165). A GenBank™ protein search (TBLASTN) of the non-redundant data base using the predicted amino acid residues identified a homologous protein named Nedd4 WW domain-binding protein 5 (N4WBP5), previously shown to bind the E3 ubiquitin ligase, Nedd4 (19). Alignment of the amino acid residues of KIAA1165 (herein after named N4WBP5A) with N4WBP5 revealed that the sequences were 51% identical and 65% similar (Fig. 1A). N4WBP5A was previously identified as part of a TBLASTN search of GenBank™ using N4WBP5 (19). In contrast to N4WBP5, which maps to human chromosome 5q31.3, the N4WBP5A genomic sequence maps to human chromosome 13q22.2 with a predicted exon/intron composition comprised of 7 exons and 6 introns (Gene Finder software, Sanger Institute, UK) (Fig. 1B, upper). N4WBP5A orthologues exist in Mus musculus, Rattus norvegicus, Bos taurus, and Xenopus laevis. A GenBank™ (TBLASTN) and HomoloGene search of N4WBP5A and N4WBP5 sequences revealed the existence of several human cDNA clones from different tissues that show homology to both proteins. Some of the cDNA clones encode proteins that appear to be truncated forms of N4WBP5A and N4WBP5. Although it is formally possible that N4WBP5A and N4WBP5 are members of a larger family of Nedd4-binding proteins, a more likely possibility is that these cDNA clones represent partial cDNA clones of either N4WBP5A or N4WBP5. Putative transcription factor binding sites within the promotor of N4WBP5A (TRANSFAC (22)) include several calcineurin targets such as NFAT-1 and Elk-1 (Fig. 1B, lower), whose known inhibition by the calcineurin inhibitors CsA and FK506 may help to explain the transcriptional control of N4WBP5A by these immunosuppressive agents.

Analysis of the amino acid sequence of N4WBP5A with an integrated search of PROSITE, Pfam, and PRINTS domain databases (23) revealed three putative transmembrane segments, two putative proline-rich WW binding domain regions, and several predicted SH2 and SH3 binding sites (Fig. 1C). Together our findings suggest the identification of a Nedd4 WW domain-binding protein.

Wide Tissue Distribution of N4WBP5A—Using RT-PCR, we cloned N4WBP5A (934 nucleotides) from human PBLs stimulated for 4 h with plate-bound anti-CD3 mAb plus PMA. The sequence of this PCR product, containing the 729-nucleotide open reading frame, was verified (BioServe Biotechnologies, Laurel, MD) to be identical to that published in GenBank™ (accession XM_041162). A random labeled [α-32P]dCTP probe for N4WBP5A (see “Materials and Methods”) was used to screen a multiple tissue Northern blot. While a single 4.4-kb transcript was expressed in the brain and lung, two transcripts (4.4 and 2.4 kb) were detected in heart, skeletal muscle, kidney, liver, and placenta (Fig. 2). Furthermore, a multiple tissue expression array confirmed the presence of N4WBP5A in the tissues mentioned above (data not shown). N4WBP5A was not detected, however, in peripheral blood for reasons that will be discussed and explored below.

N4WBP5A Localization to Golgi Complex—We subcloned the N4WBP5A cDNA, isolated from activated human PBL, into the green fluorescent protein-tagged construct, pEGFP-C3 (see “Materials and Methods”). To gain insight into the localization of N4WBP5A, HeLa and COS-7 cells were transiently transfected with either the GFP-N4WBP5A or control construct. Overexpression of the construct encoding GFP demonstrated diffuse localization, including the nucleus but excluding nucleoli. In contrast, GFP-N4WBP5A was localized in non-random distinct regions within both HeLa (Fig. 3A) and COS-7 (Fig. 3B) cells. In addition to the apparent subcellular localization of N4WBP5A, a punctate pattern of GFP-N4WBP5A localization within both cell types was noted.

To identify the subcellular compartment localized by N4WBP5A, COS-7 cells transiently transfected with GFP-N4WBP5A or control construct were stained with the Golgi complex marker, GM130, or the nuclear dye, Hoechst; cells were analyzed by immunofluorescence and confocal microscopy. The GFP-N4WBP5A localized to a subcellular compartment that overlapped with that of the Golgi complex (Fig. 4A).
In contrast, GFP-N4WBP5A subcellular localization did not overlap with the nucleus (Fig. 4B). To rule out the possibility that the subcellular distribution of N4WBP5A was due, in part, to tagging the protein with green fluorescent protein and overexpression, we assayed COS-7 cells for localization of endogenous N4WBP5A using an anti-peptide antisera. Endogenous N4WBP5A, like overexpressed GFP-N4WBP5A, was found to be localized to a subcellular compartment that overlapped with that of the Golgi complex (Fig. 4C). The localization of N4WBP5A to the Golgi complex did not explain, however, the punctate pattern in other areas of both HeLa (Fig. 3A) and COS-7 (Figs. 3B and 4).

COS-7 cells transiently transfected with GFP-N4WBP5A or control GFP construct were stained with dyes specific for either endoplasmic reticulum or mitochondria. GFP-N4WBP5A but not GFP alone co-localized, at least in part, with the endoplasmic reticulum (Fig. 5A) and mitochondria (Fig. 5B). Endogenous N4WBP5A, like overexpressed GFP-N4WBP5A, co-localized in part with mitochondria (Fig. 5C) and endoplasmic reticulum (data not shown). Together, our results suggest that N4WBP5A is localized not only to the Golgi network but also to the ER and mitochondria and in the cytoplasm of the cell.

Endogenous N4WBP5A was detected in resting (Fig. 6A) and activated human peripheral blood T cells (Fig. 6B) by immunofluorescence and confocal microscopy. Consistent with overexpressed GFP-N4WBP5A, localization of endogenous N4WBP5A was found in distinct regions as well as showing a punctate pattern throughout the cell (Fig. 6).

**N4WBP5A Association with Nedd4 via WW-1, WW-2, and WW-3 Domains**—N4WBP5A was initially identified by homology to N4WBP5, a protein that associates with the E3 ubiquitin ligase, Nedd4. To determine whether N4WBP5A also associated with Nedd4, COS-7 cells transiently transfected with GFP-N4WBP5A or GFP alone were immunoprecipitated and co-precipitating proteins immunoblotted for endogenous Nedd4. GFP-N4WBP5A, but not GFP, was able to co-immunoprecipitate Nedd4 (Fig. 7A, left panel). Immunoblotting for Nedd4 in cell lysates revealed that Nedd4 was present in both detergent-soluble and -insoluble cell fractions (Fig. 7A, right panel). We note that the pool of apparent insoluble Nedd4 was found to increase reproducibly with GFP-N4WBP5A overexpression (Fig. 7A, right panel) for reasons that are currently being explored. Immunoblotting using an anti-GFP mAb verified overexpression of GFP and GFP-N4WBP5A as indicated (Fig. 7B, left panel). While GFP-N4WBP5A was detected in both detergent-soluble and -insoluble cell fractions, a more intense band was noted in the detergent-insoluble fractions (Fig. 7B, right panel).

The amino acid sequence of N4WBP5A predicted a protein that contained two WW domain binding regions (Fig. 1C). WW domain binding regions have been shown to be important for protein-protein interactions (16, 17) and recently for the interaction between Nedd4 and N4WBP5 (19). To test whether the interaction between N4WBP5A and Nedd4 occurred via the WW domains of Nedd4, COS-7 cells transiently transfected...
with either GFP-N4WP5A or control construct were lysed and incubated with the WW-1, WW-2, and WW-3 domains of Nedd4 coupled to glutathione S-transferase (GST). GFP-N4WP5A was found to associate with all three WW domains of Nedd4 (Fig. 7C). Specificity of these interactions was suggested by the inability of GFP-N4WP5A to associate with the E3 ligase catalytic Hect domain (Fig. 7C). Our results suggest that N4WP5A associates with Nedd4 and that this interaction occurs via binding to the Nedd4 WW domains.

**N4WP5A and Nedd4 Co-localization**—Given the detected association between N4WP5A and Nedd4, we hypothesized that both proteins would co-localize to similar subcellular compartments within the cell. To test this hypothesis, we demonstrated that GFP-Nedd4 and RFP-N4WP5A ectopically co-expressed in 293T cells co-localized to similar areas of the cell (Fig. 8A). To confirm co-localization, endogenous Nedd4 and N4WP5A, like their tagged and overexpressed counterparts, were also found to localize in similar subcellular compartments (Fig. 8B). Taken together, our findings suggest that N4WP5A and Nedd4 co-localize and associate within the cell.

**Cyclosporine-sensitive Induction of N4WP5A mRNA and Protein in Human T Lymphocytes**—We generated an anti-peptide antiserum to detect endogenous N4WP5A protein (Fig. 9). Whereas the predicted molecular mass of N4WP5A is 27.1...
kDa, the antisera detected a somewhat slower migrating band at \(-29\) kDa. N4WB5A protein (Fig. 9B) and mRNA (Fig. 10A, left panel) were induced following stimulation of human PBL with anti-CD3 mAb plus anti-CD28 mAb or the calcium ionophore ionomycin, all in the presence of the protein kinase C activator, PMA. Pretreatment of the cells for 0.5 h with CsA

Fig. 8. Overexpressed and endogenous N4WB5A and Nedd4 co-localize. A. 293T cells were transiently transfected with GFP-Nedd4 and RFP-N4WB5A constructs using the CalPhos mammalian transfection procedure (see “Materials and Methods”). Detection of Nedd4 (green fluorescent protein, left panel), N4WB5A (red fluorescent protein, middle panel), and an overlay of both (right panel) is shown. B. endogenous N4WB5A was detected in untransfected 293T cells using anti-N4WB5A peptide antisera and an anti-rabbit fluorescein-conjugated secondary antibody. Endogenous Nedd4 was detected using a mouse anti-Nedd4 monoclonal antibody (BD Pharmingen, Palo Alto, CA) and a phycocerythrin-conjugated anti-mouse secondary antibody (Molecular Probes). Cells were labeled with DAPI to detect the nucleus (blue). Localization was assessed by immunofluorescence and confocal microscopy.

Fig. 9. Induction of N4WB5A protein expression in human T lymphocytes and inhibition by CsA. HeLa (A) and human PBL (A and B) whole cell lysates were prepared (see “Materials and Methods”), subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted using rabbit anti-N4WB5A antisera or pre-bleed antisera. Human PBLs were stimulated for 24 h with PMA, ionomycin, PMA plus ionomycin (PMA/Iono), anti-CD3 plus anti-CD28 plus PMA (\(\alpha\text{CD3}/\text{CD28}+\text{PMA}\)), in the presence and absence of CsA, as indicated.
prior to stimulation partially attenuated the stimulation-dependent increase of N4WBP5A protein (Fig. 9B) and mRNA (Fig. 10, left panel) expression. By contrast, N4WBP5A protein levels were not affected by PMA stimulation alone or by pretreatment of cells with CsA prior to PMA treatment (Fig. 9B). Our data suggest that N4WBP5A mRNA and protein expression are induced following T cell activation, an induction pattern that is sensitive to inhibition by the immunosuppressive agent CsA.

Differential Regulation of N4WBP5A and N4WBP5 mRNA in Human T Lymphocytes—Although N4WBP5A shares a high degree of sequence homology with the Nedd4-binding protein N4WBP5, the genes that encode these respective proteins are localized to different chromosomes. To examine whether N4WBP5A and N4WBP5 were coordinately regulated in T cells, we employed RT-PCR analyses using RNA isolated from human PBL and primers specific to each transcript. N4WBP5A mRNA levels, which were undetectable in unstimulated human PBL, were modestly induced following a 2-h treatment of cells with PMA plus ionomycin and anti-CD3/CD28 plus PMA (Fig. 10A, left panel). A robust induction of N4WBP5A was noted following a 6-h treatment of cells under similar stimulation conditions; elevated levels were maintained over the subsequent 66 h examined (Fig. 10A, left panel). The induction profile of N4WBP5, however, was markedly different than that noted for N4WBP5A. N4WBP5 mRNA levels were detected in unstimulated cells and were minimally induced upon stimulation of cells with either PMA plus ionomycin or anti-CD3/CD28 plus PMA at all time points examined (Fig. 10A, right panel). Interestingly, pretreatment of the cells with CsA inhibited...
N4WBP5A and N4WBP5 mRNA induction at 4- and 8-h post-stimulation (Fig. 10B). Inhibition of both transcripts by CsA and FK506, but not rapamycin (Fig. 10 and data not shown), implicated a common mechanism of transcriptional regulation by the serine/threonine phosphatase, calcineurin. Following 24 h of stimulation the inhibitory effects of CsA on N4WBP5A and N4WBP5 mRNA levels was abrogated. Our findings reveal disparate kinetic induction profiles for N4WBP5A and N4WBP5 in human T cells but common calcineurin-mediated regulatory pathways that modulate the gene expression of both Ned44-binding proteins.

**DISCUSSION**

We report here the cloning and initial characterization of a novel Ned44-binding protein, N4WBP5A, isolated from human T lymphocytes. We originally identified N4WBP5A as an unknown EST that was up-regulated upon T-cell activation and inhibited by the immunosuppressive agents, CsA and FK506 (20). Using RT-PCR, we show here that N4WBP5A mRNA, undetectable in resting T cells, is induced following stimulation of T cells with an activator of classic forms of protein kinase C, PM, and the calcium ionophore, ionomycin (Fig. 10). A similar induction pattern was observed upon T cell stimulation with an anti-CD3 mAb plus anti-CD28 mAb plus PMA (Fig. 10). Although the amino acid sequence analysis of N4WBP5A revealed high homology with the Ned44-binding protein N4WBP5 (Fig. 1A), we show here that transcriptional regulation of these proteins differed. Unlike N4WBP5A, N4WBP5 was present in resting T cells and only modestly induced following activation (Fig. 10). The induction of both N4WBP5A and N4WBP5 mRNA was sensitive to CsA and FK506 (Fig. 10), but not rapamycin (data not shown), suggesting that the serine/threonine phosphatase calcineurin regulated expression. Analysis of the proximal promoter regions of N4WBP5A (Fig. 1B) and N4WBP5 (data not shown) revealed several putative transcription factor binding sites, capable of binding factors regulated by calcineurin; analyses of the proximal promoters are a subject of current study. We have not ruled out the possibility that the transcription factors that regulate N4WBP5A and N4WBP5 may be different and yet sensitive to calcineurin.

We found that N4WBP5A localized predominantly, but not exclusively, to the Golgi complex (Fig. 4). Overexpressed and endogenous N4WBP5A also localized to other cellular organelles, including the ER and mitochondria (Fig. 5 and data not shown); the subcellular localization of cytoplasmic endogenous N4WBP5A in T cells could not be further resolved (Fig. 6). Amino acid sequence analysis revealed that N4WBP5A has three putative transmembrane segments suggesting that N4WBP5A is membrane-localized, and consistent with this prediction, GFP-N4WBP5A was found predominantly in the detergent-insoluble pellet (Fig. 7B). Taken together, our data suggest that N4WBP5A is embedded within the membrane of the Golgi complex and other organelles and interacts with Ned44. Our findings that Ned44 and N4WBP5A co-localize within the cell (Fig. 8) and that an increase in the pool of insoluble Ned44 is noted with N4WBP5A overexpression (Fig. 7A, right panel) are consistent with this model.

The N4WBP5A amino acid sequence analysis revealed two proline-rich WW domain binding regions (Fig. 1C) capable of binding Ned44 (Fig. 7). We show that the interaction of N4WBP5A with Ned44 (Fig. 7A) was mediated by the Ned44 WW-1, WW-2, and WW-3 domains (Fig. 7C). While N4WBP5A and Ned44 co-immunoprecipitate, it is possible that other proteins interact in a multiprotein complex. The N4WBP5A association with Ned44 raises the compelling question as to whether N4WBP5A regulates the activity of Ned44 (or other WW domain-containing proteins) or affects the ubiquitination and turnover of the Ned44 substrate, ENaC. In parallel, we cannot rule out the possibility that N4WBP5A may itself be a target of Ned44, flagged for ubiquitination and proteasome-mediated degradation. No function for Ned44 has yet been identified in T lymphocytes. A murine Ned44 family member, Itch, has been shown to be disrupted in non-agouti lethal mice (Itch mice), resulting in abnormal immune responses and an epidermal inflammatory disorder (24, 25). Recently Itch was also shown to be involved in T lymphocyte regulation and implicated in Th2 differentiation (5). In addition to Itch, other WW-containing proteins and other E3 ubiquitin ligases (e.g. c-cbl, cbl-b, AIP-4, etc.) have been shown to be important in lymphocyte function (26). We are therefore currently investigating a potential association between N4WBP5A and these other molecules.

In the course of submitting the manuscript for this report, Konstas and coworkers (21) reported that overexpression of N4WBP5A in X. laevis oocytes results in an increase in the cell surface expression of the epithelial sodium channel (ENaC) and prevents sodium feedback inhibition of ENaC. Importantly, they further demonstrated that endogenous N4WBP5A associates with Ned44 and Ned44-2 in a murine cortical duct cell line. Given the research interests of our laboratory, we have explored the role of N4WBP5A in T cells. In T lymphocytes N4WBP5A co-localizes with Ned44; the one or more binding partners of Ned44 are still unknown. We found further that N4WBP5A mRNA is induced following T cell engagement in a cyclosporine-sensitive manner (Fig. 10).

The Northern blot (Fig. 2) and tissue expression array (data not shown) did not detect N4WBP5A in peripheral blood, consistent with the RT-PCR analysis (Fig. 10). However, N4WBP5A was expressed as a single 4.4-kb transcript in brain and lung and as two transcripts (4.4 and 2.4 kb) in heart, skeletal muscle, kidney, liver, and placenta (Fig. 2). It will be interesting to determine whether N4WBP5A mRNA is regulated by calcineurin in other tissues (e.g. brain and kidney) and, importantly, whether it plays a role in the toxicities (e.g. nephrotoxicity and neurotoxicity) observed clinically with cyclosporine and tacrolimus (FK506) treatment.

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**REFERENCES**

1. Ben-Neriah, Y. (2002) Nat. Immunol. 3, 20–26
2. Rao, N., Dodge, L., and Band, H. (2002) J. Leukoc. Biol. 71, 753–763
3. Ciechanover, A., Orian, A., and Schwartz, A. L. (2000) J. Cell. Biochem. Suppl. 34, 40–51
4. Rotin, D., Staub, O., and Huguenauer-Tsapis, R. (2000) J. Membr. Biol. 176, 1–17
5. Patel, P., Mohammed, R., and Stuehr, D., (1999) J. Biol. Chem. 274, 32336–32346
6. Treier, M., Staszewski, L., and Bohmann, D. (1999) Mol. Cell 78, 579–787, 1994
7. Spataro, V., Nurbury, C., and Harris, A. L. (1998) Br. J. Cancer 77, 448–455
8. Plant, P. J., Yeger, H., Staub, O., Howard, P., and Rotin, D. (1997) J. Biol. Chem. 272, 52529–52536
9. Suzuki, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7753–7758
10. Kumar, S., Harvey, K. F., Kinoshita, M., Copeland, N. G., Noda, M., and Jenkins, N. A. (1995) EMBO J. 14, 43596–43602
11. Chen, H. I., and Sudol, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7819–7823
12. Sudol, M., Chen, H. I., Boegger, C., Einhorn, A., and Bork, P. (1995) FEBS Lett. 369, 67–71
13. Joliffe, C. N., Harvey, K. F., Haines, B. P., ParasAVIS, G., and Kumar, S.
19. Harvey, K. F., Shearwin-Whyatt, L. M., Fotia, A., Parton, R. G., and Kumar, S. (2002) J. Biol. Chem. 277, 9307–9317
20. Cristillo, A. D., and Bierer, B. E. (2002) J. Biol. Chem. 277, 4465–4476
21. Konstas, A. A., Shearwin-Whyatt, L. M., Fotia, A. B., Degger, B., Riccardi, D., Cook, D. I., Korbmacher, C., and Kumar, S. (2002) J. Biol. Chem. 277, 29406–29416
22. Prestridge, D. S. (1991) Comput. Appl. Sci. 7, 203–206
23. Apweiler, R., Biswas, M., Fleischmann, W., Kanapin, A., Karavidopoulou, Y., Kersey, P., Kriventseva, E. V., Mittard, V., Mulder, N., Phan, I., and Zdobnov, E. (2001) Nucleic Acids Res. 29, 44–48
24. Perry, W. L., Hustad, C. M., Swing, D. A., O’Sullivan, T. N., Jenkins, N. A., and Copeland, N. G. (1998) Nat. Genet. 18, 143–146
25. Qiu, L., Joazeiro, C., Fang, N., Wang, H.-Y., Elly, C., Altman, Y., Fang, D., Hunter, T., and Liu, Y. C. (2000) J. Biol. Chem. 275, 35734–35737
26. Liu, Y. C., and Gu, H. (2002) Trends Immunol. 23, 140–143