Analysis of Nucleocytoplasmic Trafficking of the HuR Ligand APRIL and Its Influence on CD83 Expression

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Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system and are able to sensitize even naïve T cells. Mature DC are characterized by expression of CD83, a surface molecule that is proposed to be involved in efficient T cell activation. It has been recently shown that CD83 mRNA is transported from the nucleus to the cytoplasm in a HuR- and CRM1-dependent manner. Therefore we here investigated the impact of two known protein ligands of HuR, pp32 and APRIL, on CD83 expression. Both pp32 (ANP32A) and APRIL (ANP32B) are shuttle proteins, and it has been reported earlier that these HuR ligands can act as adaptors that link HuR and the CRM1-specific nuclear export pathway. By employing RNA interference (RNAi) technology we demonstrate that pp32 is dispensable for CD83 expression, whereas APRIL contributes to the nuclear export and subsequent translation of CD83 mRNA. Furthermore, we have determined the nuclear import signal (NLS) as well as the nuclear export signal (NES) of human APRIL. Moreover, we analyzed the status of phosphorylation of endogenous APRIL and identified threonine 244 to be an as yet unrecognized phosphate acceptor. Finally, we were able to show that phosphorylation of this specific amino acid residue regulates the nuclear export of APRIL. In sum, we report here the signal sequences in APRIL that mediate its intracellular trafficking and provide evidence that this protein ligand of HuR is an important player in the post-transcriptional regulation of CD83 expression by affecting the nucleocytoplasmic translocation of CD83 mRNA.

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the immune system that are specialized to induce T cell-mediated immunity and are able to activate even naïve CD4+ and CD8+ T cells (1–4). Therefore DC are frequently referred to as nature’s adjuvant. Immature DC reside in peripheral tissues. Following uptake of antigen and exposure to inflammatory stimuli, they migrate to the secondary lymphoid organs, where, as mature DC, they can initiate antigen-specific T cell immune responses. The DC maturation process includes the modulation of endogenous chemokine and chemokine receptor expression profiles, as well as the up-regulation of several cytokines, costimulatory, and adhesion molecules (1–4).

CD83 is expressed on the surface of mature DC and, to a lower extent, also on T and B cells (5–7). Although its exact function remains to be determined, accumulating data support the model that CD83 contributes to efficient T cell activation (reviewed in Refs. 8–10). For example, competition of endogenous CD83 by addition of soluble forms of CD83 abrogates DC-mediated T cell activation in vitro as well as in mice (11, 12). Therefore, the detailed analysis of the processes that regulate CD83 expression will not only improve our understanding of T cell activation, but may also result in the identification of novel drug targets that can potentially be exploited to modulate DC activity.

In metazoans, the vast majority of mRNAs are transported from the nuclear site of transcription to the cytoplasm, the site of protein synthesis, via the TAP/NXF1 pathway (reviewed in Refs. 13–15). In contrast, it has been recently shown that a small subpopulation of cellular transcripts, including the mRNA encoding CD83, can be translocated across the nuclear envelope via the completely unrelated CRM1 nuclear export pathway (16). In another study, a novel cis-active RNA element, termed post-transcriptional regulatory element (PRE), which serves as binding site of the HuR nucleocytoplasmic shuttle protein, has been identified in CD83 mRNA (17). In contrast to the binding of HuR to AU-rich elements (AREs), which are frequently located in the 3'-untranslated region of early response gene (ERG) mRNAs (18), the interaction of HuR with the CD83 PRE, which is located in the gene coding region, does not result in RNA stabilization, but rather mediates the nuclear export of this transcript. In this context it is interesting to note that Steitz and co-workers (19, 20) previously provided evidence that HuR-dependent nuclear export of the ARE-containing c-fos mRNA is mediated by the ANP32 family members pp32 (ANP32a) and/or APRIL (ANP32b), which appear to interfering RNA; UTR, untranslated region; wt, wild type; MOPS, 4-morpholinepropanesulfonic acid.
serve as adaptor molecules, linking HuR and the nuclear export receptor CRM1. Therefore we hypothesized that the HuR ligands pp32 and/or APRIL may also play an important functional role in the nucleocytoplasmic translocation of CD83 mRNA.

Here we analyzed the effect of pp32 and APRIL on CD83 expression. We demonstrate that the HuR ligand APRIL, but not pp32, regulates the cytoplasmic accumulation of CD83 transcripts and characterize the nucleocytoplasmic shuttling of APRIL in detail.

**MATERIALS AND METHODS**

**Molecular Clones**—Wild-type and mutant genes were inserted into various vectors using standard methods and synthetic double strand oligonucleotides or PCR technology. Because of extensive sequence homology, human pp32 and APRIL sequences were pre-amplified from a cDNA derived from peripheral blood mononuclear cells (PBMC) with primers binding to the untranslated regions of the respective transcript (21, 22): pp32 5’-CCTCTGCAGAGAGAGACG-3’ (forward), 5’-GTCACTCCTTTCCCACTTC-3’ (reverse); APRIL 5’-GGAGTGGTTAAGTTAAGAGG-3’ (forward), 5’-CTGTTTCTCAGGTTACAGTG-3’ (reverse). In combination with appropriate primers, the obtained products were subsequently used as templates for the final amplification of the pp32 (21) or APRIL (22) coding region by PCR. The plasmids pGEX-APRIL and pGEX-pp32 are bacterial expression vectors in which the human APRIL or the human pp32 cDNA are fused in-frame to the 3’-end of the glutathione S-transferase (GST) coding region by inserting the respective sequences between the EcoRI and BamHI or EcoRI and XhoI site of pGEX-5X-1 (Amersham Biosciences), respectively. The eukaryotic expression vectors p3pp32-FLAG and p3APRIL-FLAG were created by fusing the respective sequences between the EcoRI and BamHI or EcoRI and XhoI site of pGEX-5X-1 (Amersham Biosciences). The subsequent mutational inactivation of the binding to the untranslated regions of the respective transcript (25, 26).

**RNA Isolation, Slot-blot Analysis, and PCR**—Total cellular RNAs were isolated according to the manufacturer’s protocol by using TRIzol reagent (Invitrogen). For isolation of cytoplasmic and nuclear RNAs, 2 × 10^6 cells were lysed on ice for 1 min using 100 μl of Nonidet P-40 buffer (10 mM Hepes-KOH pH 7.8, 10 mM KCl, 20% glycerol, 1 mM dithiothreitol, 0.25% Nonidet P-40). Subsequently, the lysates were cleared by centrifugation at 470 × g for 5 min at 4 °C. Cytoplasmic RNA was isolated from 80 μl of the supernatant using TRIZol reagent. The nuclei were washed again in 100 μl of Nonidet P-40 buffer to deplete residual cytoplasmic RNA. Afterward, the nuclear RNA was isolated by using TRIzol reagent. If necessary, DNase treatment of the RNA samples was performed. Selected RNA samples were reversely transcribed using the first strand cDNA (AMV) synthesis kit for RT-PCR (Roche Applied Science) according to the manufacturer’s instructions. Subsequently, RT products were assayed by PCR. For detection of GADPH sequences following primers were used: forward, 5’-TGAAGGTCGGAATCCAGTTTG-3’; reverse, 5’-CATGTTGGGCCATGAGGTCAC-3’.

The amplification profile involved 25 cycles of denaturation at 95 °C for 1 min, primer annealing at 56 °C for 1 min, and primer extension at 72 °C for 1 min.

CD83 RNAs were detected by using following primer pairs: forward, 5’-GGTGAAGGTGGCTTGCTCCGAAG-3’, and reverse, 5’-GAGCCAGCAGGACAATCTCC-3’. The amplification profile involved 25 cycles of denaturation at 95 °C for 1 min, primer annealing at 56 °C for 1 min, and primer extension at 72 °C for 1 min. Real time PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification was monitored by SYBR green fluorescence. Standard curves were derived by serial dilution of p3UTR-CD83 and pGADPH. RNAs were normalized by amplification of GADPH.

For immuno-PCR analysis, transiently expressed APRIL-FLAG protein was covalently cross-linked to HuR-CD83 mRNA complex by adding 1% formaldehyde to the medium for 5 min at 37 °C. Afterward, FLAG-tagged APRIL was immunoprecipitated with a monoclonal anti-FLAG-antibody. Coimmunoprecipitated CD83 mRNA was reverse-transcribed and analyzed by PCR as described above.
Nucleocytoplasmic Trafficking of APRIL

For RNA fractionation control 10 μl of 0.1 μg/μl total, nuclear or cytoplasmic RNA were supplemented with 30 μl of RNA denaturation buffer (660 μl of formamide, 210 μl of 37% formaldehyde, 130 μl of 10× MOPS electrophoresis buffer, pH 7.0) and incubated for 5 min at 65 °C. Afterward, the samples were cooled on ice and supplemented with 40 μl of 20× SSC. Positively charged Hybond-N membrane (Amersham Biosciences) was preincubated in 20× SSC and placed on a slot-blotting Miniifold II system (Whatman). The RNA samples were applied to the slots by vacuum soaking, and the membrane was subsequently washed with 500 μl of 10× SSC per slot. Afterward, the RNA was covalently cross-linked by UV irradiation (UV-Stratalinker 1800; Stratagene), and the membrane was subjected to Northern blot analysis described elsewhere (29) using a radioactive labeled antisense U6 snRNA-specific oligonucleotide (5’-GAACGCTTCAGAATTTGCGT-3’).

Antibodies—Various Cys-KLH-coupled peptides were used to generate specific antibodies in rabbits: pp32, NH2-DEEG-YNDGEVDDEE-COOH (amino acid positions 215–227 (22); APRIL phosphospecific-Thr 244,N H2-GEKRKREpTDDEGEDD-COOH (amino acid positions 237–251 (22). The anti-HuR polyclonal antisera and the monoclonal antibody recognizing hnRNA A1 (4B10) have been described (33) using virus-containing supernatants equivalent to generate specific antibodies in rabbits: pp32, NH2-DEEG-YNDGEVDDEE-COOH (amino acid positions 215–227 (22); APRIL phosphospecific-Thr 244,N H2-GEKRKREpTDDEGEDD-COOH (amino acid positions 237–251 (22). The anti-HuR polyclonal antisera and the monoclonal antibody recognizing hnRNA A1 (4B10) have been described (33) using virus-containing supernatants equivalent

Protein Analyses—Total cellular protein was isolated by lysis of the cells with lysis buffer A (0.1% Nonidet P-40, 150 mM NaCl, 50 mM Hepes at pH 7.3, 1 mM EDTA, 4 mM EGTA, 5 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 125 μg/ml Pefablock (Biomol)) followed by denaturation in SDS buffer (65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1.5% dithiothreitol). For isolation of cytoplasmic and nuclear proteins 2 × 106 cells were lysed on ice for 1 min using 100 μl of Nonidet P-40-buffer (10 mM Hepes-KOH pH 7.8, 10 mM KCl, 20% glycerol, 1 mM dithiothreitol, 0.25% Nonidet P-40). Subsequently, the lysates were cleared by centrifugation at 470 × g for 5 min at 4 °C. Cytoplasmic protein was isolated from 80 μl of the supernatant and denatured in SDS buffer. The nuclei were washed again in 100 μl of Nonidet P-40-buffer to deplete residual cytoplasmic proteins. Afterward, the nuclear proteins were prepared by denaturation of the nuclei in SDS buffer. Immunoprecipitation analysis, SDS-PAGE, and Western blot analysis were performed as previously described (17, 34).

Protein Purification—GST-APRIL and GST-pp32 was expressed in Escherichia coli BL21(DE3). Accordingly, 500-ml cultures were grown to an A600 of ~0.5, and protein expression was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside to the respective culture media. After incubation at 30 °C for 3 h, bacteria were harvested and lysed in 20 ml of lysis buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 125 μg/ml Pefablock (Biomol), 3 mg lysozyme). The lysate was cleared by centrifugation and applied to 2 ml of glutathione-Sepharose 4B (Amersham Biosciences) at 4 °C for 30 min. The Sepharose was washed three times with wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.4% Nonidet P-40) and bound GST proteins were eluted with 20 mM glutathione (dissolved in wash buffer). After extensive dialysis against PBS/20% glycerol, the proteins were subjected to further analyses.

Metabolic Labeling and Pulse-Chase Experiments—Cells were washed twice with PBS and incubated with cysteine/methionine-free medium containing 10% dialyzed fetal calf serum for 1 h. The cells were metabolically labeled for 30 min using 500 μCi of Tran35S-label (MP Biochemicals; 1175 Ci/mmol). Afterward the cultures were washed twice with PBS and either harvested or, in case of pulse-chase experiments, incubated further for various time periods in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (penicillin and streptomycin). For deglycosylation the cellular lysates were incubated with PNGase F (New England Biolabs) according to the manufacturer’s instructions.

Indirect Immunofluorescence Microscopy—For indirect immunofluorescence analysis cells were grown on coverslips and subsequently fixed with 3% paraformaldehyde for 25 min at ambient temperature. After incubation in 100 mM glycine/PBS for 10 min, the cells were permeabilized in PBS/0.1% Triton X-100 for 4 min at ambient temperature, followed by incubation in
PBS/1% BSA for 30 min. Proteins were stained in PBS/1% BSA for 30 min with mouse monoclonal antibodies directed against βGal (1:200 dilution) or the FLAG epitope (1:5,000 dilution), followed by Alexa-Fluor-594-coupled anti-mouse antibody raised in goats (1:800 dilution, Molecular Probes). Nuclei were visualized by Hoechst 33258 (0.25 μg/ml in PBS) staining for 10 min. The cells were mounted in mowiol medium and analyzed using a Zeiss Axiovert-200 M microscope.

**Heterokaryon Assay**—HeLa cells were seeded at day 1 post-transfection together with untransfected NIH 3T3 cells on coverslips. The cocultures, supplemented with 75 μg/ml cycloheximide to block de novo protein synthesis, were then further incubated for 2 h. Heterokaryon formation was induced by exposure of the cell cultures to 50% polyethylene glycol 3350 in PBS for 2 min at 37 °C. The coverslips were then washed extensively in PBS and returned to fresh culture medium containing 75 μg/ml cycloheximide. After incubation for another hour, cells were fixed and indirect immunofluorescence analysis was performed.

**RESULTS**

**Analysis of pp32 and APRIL Expression**—In a previous study, we provided evidence that HuR is required for CD83 expression in Jurkat T cells (17). Therefore we analyzed here the expression level of the HuR ligands pp32 and APRIL in logarithmically growing Jurkat T cells. For this, we raised pp32- and APRIL-specific polyclonal antibodies in rabbits. At first, the respective sera were tested in Western blot analyses for their capacity to recognize purified pp32 or APRIL GST fusion proteins. As shown, both sera were highly specific and recognized their targets with similar affinities (Fig. 1A, lane 7 versus lane 8), allowing us to distinguish the closely related pp32 and APRIL proteins in this type of assay. The subsequent detection of the respective endogenous proteins in Jurkat cells revealed that the steady state expression level of APRIL is significantly higher than the level of pp32 in these cells (Fig. 1B, compare lane 1 and lane 2). To analyze, whether this difference is caused by protein stability, a protein pulse-chase experiment was performed (Fig. 1C). These results clearly showed that APRIL is more stable (half-life ~ 28 h) than pp32 (half-life ~ 12.5 h). Remarkably, the analysis of the respective relative mRNA levels by real time PCR revealed that pp32 transcripts are more abundant in Jurkat cells than mRNAs encoding APRIL (data not shown). Thus, the observed differences in protein half-life most likely explain the different steady state expression levels of these two HuR ligands observed in Jurkat T cells.

**Silencing of APRIL Abrogates Cytoplasmic Accumulation of CD83 mRNA**—Because it has been shown that both pp32 and APRIL appear to be complexed with HuR and CRM1 (19, 20), we investigated the impact of these ANP32-family members on CD83 mRNA transport and protein expression in a heterologous approach. As shown by Western blot analyses, transfection of COS cells with a pp32- or APRIL-specific short hairpin (sh)RNA expression vector (pshpp32 or pshAPRIL, respectively) decreased clearly the level of the respective endogenous proteins (Fig. 2A). Next, COS cells were cotransfected with a plasmid expressing the CD83 cDNA flanked by the entire homologous 5’- and 3’-UTR (p3UTR-CD83, depicted in Fig. 2B) together with either the pp32-specific or the APRIL-specific shRNA vector, respectively. Three days post-transfection, the transfected cultures were metabolically labeled with Tran35S-label and de novo CD83 synthesis was determined by CD83-specific immunoprecipitation analysis. Unexpectedly, these experiments demonstrated that RNAi-mediated knockdown of pp32 does not influence CD83 synthesis (Fig. 2B, lane 2 versus lane 3). In sharp contrast, however, knockdown of APRIL resulted in significant down-regulation of CD83 expression in these transfected cells (Fig. 2B, lane 5 versus lane 6).

Because APRIL can act as an adapter molecule in CRM1-dependent nuclear export of HuR (19, 20), and the HuR-CRM1 axis has been shown to be operational in the nucleocytoplasmic transport of CD83 mRNA (16, 17), we hypothesized that the observed impact of APRIL on CD83 expression may be caused by affecting nuclear export of CD83 mRNA. To test this hypothesis, COS cells were transfected with pshAPRIL and, 3 days later, with the CD83 expression vector p3UTR-CD83. After another day of culturing, the total, cytoplasmic, and nuclear RNA was isolated from these cells for subsequent RT-PCR analysis. The fractionation method was controlled by detection of the nuclear U6 snRNA and demonstrated that this specific RNA is, upon cellular fractionation, significantly more abundant in the nucleus as compared with the cytoplasm. The combined experiments revealed that APRIL knockdown did not significantly alter the abundance of CD83 messages in total cellular RNA (Fig. 2C, upper panel, lane 1 and lane 2). However, the silencing of APRIL clearly decreased the cytoplasmic CD83 mRNA level (Fig. 2C, upper panel, compare lane 3 and lane 4), which was accompanied by increased accumulation of this specific transcript in the nucleus (Fig. 2C, upper panel, compare lane 5 and lane 6). To confirm these findings in a more quantitative fashion we also performed real time PCR. This analysis closely mirrored the previous result, suggesting that APRIL is required for efficient cytoplasmic accumulation of CD83 mRNAs (Fig. 2C, lower panel).

As silencing of APRIL clearly diminished the cytoplasmic accumulation of CD83 mRNA in heterologous transfected cell cultures, we next questioned whether APRIL also affects the intracellular distribution of authentic endogenous CD83 mRNA. We therefore infected Jurkat T cells with lentiviral pseudotypes that either expressed shRNA directed toward APRIL mRNA or off-target shRNA for control (directed toward luciferase mRNA). Five days after infection, the Jurkat cells were stimulated with PMA and ionomycin to activate CD83 expression (35) and the subcellular distribution of the endogenous CD83 mRNA was subsequently analyzed as before. These experiments confirmed our data obtained in transiently transfected cells, demonstrating a decreased level of cytoplasmic CD83 mRNA upon silencing of APRIL (Fig. 2D, upper panel, compare lane 3 and lane 4), whereas the total and nuclear level of endogenous CD83 mRNA remained largely unaffected (Fig. 2D, upper panel, lanes 1, 2, 5, and 6). Again, comparable results were obtained by quantitative real time PCR analysis (Fig. 2D, lower panel).

Next we wanted to demonstrate that APRIL associates with both, HuR and CD83 mRNA. Therefore, COS cells were cotransfected with various combinations of p3UTR-CD83,
p3APRIL-FLAG, and pBC12/CMV/HuR. At 24 h post-transfection a chemical cross-linker (1% formaldehyde) was added to the cultures and APRIL (FLAG)-specific immunoprecipitation was performed using the respective total cell extracts. Subsequently, the corresponding precipitates were subjected to RT-PCR using CD83-specific oligonucleotides. As shown, CD83 mRNA was only detectable in presence of APRIL and HuR in these experiments (Fig. 2E). The combined data therefore demonstrate that the HuR-ligand APRIL is involved in the nucleocytoplasmic translocation of CD83 mRNA.

FIGURE 1. Different steady-state levels of pp32 and APRIL correlate with protein half-life. A, analysis of pp32- and APRIL-specific polyclonal antipeptide antisera. 0.5 μg of recombinant and purified GST-pp32 or GST-APRIL, respectively, was separated by SDS-PAGE. Western blot analyses were performed by using GST-specific antibody or rabbit serum recognizing pp32 and/or APRIL as indicated (anti-GST, anti-pp32, anti-APRIL). B, analysis of endogenous pp32 and APRIL levels in Jurkat T cells. Crude Jurkat extracts (20 μg) were separated by SDS-PAGE. Western blot analysis was performed to detect pp32 (lane 1), APRIL (lane 2) and α-tubulin (gel loading control), respectively. C, analysis of protein half-lives. 1 × 10⁷ asynchronous, logarithmically growing Jurkat T cells were metabolically labeled using Tram35S-label for 30 min. Afterward, the cultures were washed twice with PBS, and RPMI medium was added. Cells were harvested at the indicated time points and immunoprecipitation of pp32 or APRIL was performed, using the antisera described in A. Experiments were performed in duplicates and representative IPs are displayed. Quantification of the obtained signals by phosphorimage analysis to determine the half-lives of pp32 (upper panel) and APRIL (lower panel) is shown.
Nucleocytoplasmic Trafficking of APRIL

A Short Basic Amino Acid Sequence Serves as Nuclear Localization Signal of APRIL—Because pp32 seems to be dispensable for CD83 expression, our further investigation focused now on the regulation of the intracellular transport of APRIL. APRIL has been shown to shuttle between the nucleus and cytoplasm and, therefore, must contain signal sequences that direct its nuclear import and nuclear export (19, 22). To first define the sequences in APRIL that mediate its nuclear import, we performed subcellular localization studies. For this, we tested various APRIL derivatives by indirect immunofluorescence microscopy for their ability to target the cytoplasmic β-galactosidase (βGal) reporter protein to the cell nucleus. This analysis revealed that the C-terminal region of APRIL contains a nuclear localization signal (NLS; data not shown). This NLS was subsequently mapped to a short stretch of basic amino acid residues at position 239–242 in APRIL. As shown, βGal localized in the cytoplasm (Fig. 3, A and B), but was directed into the nucleus when it was expressed as a fusion to APRIL wild type (wt) protein (Fig. 3, C and D). Nuclear accumulation was abrogated when the basic amino acids at position 239–242 (NH₂₁KRKR-COOH) in APRIL were substituted by alanines (APRIL-NLS mut; Fig. 3, E and F), indicating that these residues constitute the NLS of human APRIL. NLS activity of this sequence was further confirmed by demonstrating that these four amino acids are sufficient to cause efficient nuclear accumulation of otherwise cytoplasmic βGal (Fig. 3, G and H).

A Distinct NES Mutant Abrogates Nucleocytoplasmic Shuttling of APRIL—On basis of a sequence comparison it has been previously suggested that APRIL contains three putative NES that resemble the canonical leucine-rich NES of the Rev trans-activator of human immunodeficiency virus type 1 (HIV-1) (19). To date, however, these putative NESs (here referred...

FIGURE 2. APRIL-dependent cytoplasmic accumulation of CD83 mRNA. A, COS cells were transfected either with the pshpp32 (lane 2), the pshAPRIL (lane 4), or the parental pSUPER (control; lanes 1 and 3) vector. Three days post-transfection, the cell cultures were harvested and the respective protein extracts (20 μg) were subjected to pp32-, APRIL-, or α-tubulin-specific (gel loading control) Western blot analyses as before. The efficiencies of the respective knockdowns were quantified and are indicated below the Western blots. B, upper, schematic depiction of the expression vector p3UTR-CD83 encoding for human CD83 cDNA flanked by the entire homologous 5' - and 3' -UTR. CMV-IE, cytomegalovirus immediate early promoter; CDS, coding sequence; pA, polyadenylation site. Lower, COS cells were either mock-transfected or cotransfected with the CD83 expression vector p3UTR-CD83 together with the parental pSUPER plasmid (control) or the indicated shRNA vectors (pshpp32 or pshAPRIL, respectively). At day 3 post-transfection, cell cultures were radiolabeled, and the respective cellular extracts were subjected to CD83-specific immunoprecipitation, followed by SDS-PAGE. CD83 protein was detected by autoradiography and the obtained signals were quantified by phosphorimage analysis (indicated in percent). In addition, each radiolabeled extract was controlled by α-tubulin-specific Western blot analysis. C, upper, COS cells were transfected with p3UTR-CD83 and pshAPRIL (+) or pSUPER (−; control) as before. Total (T), cytoplasmic (C), and nuclear (N) RNA was isolated and the abundance of CD83 mRNA was analyzed by RT-PCR. Detection of transcripts encoding GAPDH served as control. The fractionation method was controlled by detection of U6 snRNA using a slot-blot device. Lower, quantification of the CD83-specific transcripts by real time PCR analysis. The results are expressed as ratios of CD83 mRNA in cells (−/+ silencing of APRIL (normalized to transcripts encoding GAPDH). D, upper, Jurkat T cells were infected with lentiviral pseudotypes expressing either APRIL-specific (+) shRNA or off-target shRNA (−). The respective Jurkat cultures were stimulated with PMA/ionomycin at day 5 postinfection and total (T), cytoplasmic (C), and nuclear (N) RNA was subsequently isolated for RT-PCR. Lower, quantification of the CD83-specific transcripts by real time PCR as before. E, COS cells were cotransfected as indicated with p3APRIL-FLAG, pBc12/CMV/HuR and p3UTR-CD83. At 24 h post-transfection the cultures were supplemented for 5 min to 1% formaldehyde. Subsequently, the respective total cellular extracts were subjected to APRIL-specific immunoprecipitation using anti-FLAG antibodies and analyzed by CD83-specific RT-PCR.
Nucleocytoplasmic Trafficking of APRIL

FIGURE 3. Mapping of the nuclear localization signal (NLS) in APRIL. HeLa cell cultures were transiently transfected with constructs expressing the indicated fusion proteins. Two days posttransfection, cells were fixed with paraformaldehyde and indirect immunofluorescence analysis was performed to determine the subcellular localization of βGal and βGal-APRIL fusion proteins (corresponding images are arranged side by side). Nuclei were visualized by Hoechst 33258 staining (panels A, C, E, and G) and the transiently expressed proteins by using βGal-specific antibody (panels B, D, F, and H). A four amino acid sequence, KRKR (amino acids 239–242) serves as unique NLS of APRIL and is able to carry the cytoplasmic β-galactosidase protein to the nucleus. Hela cells were transfected with expression vectors encoding for βGal-APRIL-wt fusion protein (panels A and D), βGal-APRIL NLSmut fusion protein (E and F), βGal (A and B), and βGal-NLS fusion protein (G and H), respectively.

to NES1–3 for technical reasons) have not been functionally analyzed.

To identify the NES sequences in APRIL, we decided to generate epitope (FLAG)-tagged APRIL mutant proteins, in which several leucine to alanine substitutions disrupt the putative NES sequences (NES1–3). The nuclear export ability of these APRIL mutants was tested in standard heterokaryon assays, in which transiently transfected human HeLa cells (expressing the various APRIL variants) were fused with untransfected mouse NIH 3T3 cells to form heterokaryons. Fixed cells were analyzed by indirect immunofluorescence analysis using FLAG- and βGal-specific antibodies. The cultures were also treated with Hoechst 33258 dye, which allows us to distinguish human and mouse nuclei (the latter are characterized by a typical speckle pattern). As shuttling-positive internal control, an expression vector encoding GFP-Rev fusion protein was cotransfected in each experiment. Furthermore, transfection of the NES-deficient βGal-NLS construct (see Fig. 3) served as shuttling negative control. As expected, the GFP-Rev fusion protein was able to migrate from the human (indicated by arrows) to the mouse nucleus in these experiments (Fig. 4, panels B, E, H, L, and O, respectively) and the βGal-NLS fusion did not leave the HeLa cell nucleus (Fig. 4C). In agreement with a previous study (19), the APRIL wt protein clearly displayed shuttling activity, as demonstrated by its obvious accumulation in mouse nuclei (Fig. 4F). Likewise, the APRIL mutants, in which the putative NES1 (amino acid position 60–71) or NES2 (amino acid position 83–95) was inactivated, were transported from human to mouse nuclei, indistinguishable from the wild-type protein (Fig. 4I and M, respectively). In sharp contrast, however, mutation of the Rev-like NES3 at APRIL amino acid positions 109–120 (NH2-LKK-LECLKSLDL-COOH) prevented nucleocytoplasmic shuttling of the respective protein, indicating that this sequence indeed executes NES activity (Fig. 4P).

Nuclear Export of APRIL Is Regulated by Phosphorylation of Thr244 —Like other members of the ANP32 family, human APRIL is a putative phosphoprotein (reviewed in Ref. 36). In silico calculation using a computer program of the Centre of Biological Sequence Analysis of the Technical University of Denmark (TDU) was therefore performed to predict the putative phosphorylation sites Ser72, Ser104, Ser158, Ser194, and Thr244 in human APRIL (Fig. 5A). To directly investigate its phosphorylation status, endogenous APRIL of logarithmically growing HSC93 cells (a human B cell lymphoma) was isolated by immunoaffinity chromatography, digested with trypsin and subjected to MALDI-TOF mass spectrometry (MS) analysis as previously described (34) (data not shown). Unfortunately, the central region of APRIL, accommodating three putative phosphorylation sites (Tyr148, Ser158, and Ser210), repeatedly resisted any sequencing attempt. None the less, we were able to demonstrate that two putative phosphorylation sites (Ser72, Ser104) are not phosphorylated under these experimental conditions. In contrast, the threonine residue at amino acid position 244, located in close vicinity of the APRIL NLS (amino acids 239–242), was found to be partially phosphorylated (summarized in Fig. 5A).

We next generated an antisera (anti-pT244) that specifically recognizes Thr244-phosphorylated APRIL by immunizing rabbits with a Thr244-phosphorylated peptide. The specificity of this serum was verified by analyzing recombinant GST-APRIL fusion protein isolated from E. coli (unphosphorylated form) and B-cell-derived native APRIL (phosphorylated form). As shown, our former anti-APRIL antiserum recognized both bacterial expressed as well as B cell-derived APRIL (Fig. 5B, lanes 1 and 2). By contrast, particularly the affinity-purified anti-pT244 antisera recognized specifically the phosphorylated form of APRIL (Fig. 5B, compare lanes 5 and 6). In addition, we also generated an APRIL mutant, in which Thr244 was substituted by an alanine residue (APRIL T244A). Expression of FLAG-tagged APRIL variants in COS cells and subsequent Western blot analyses revealed that anti-pT244 antibodies recognize wild-type APRIL, but not APRIL T244A mutant protein (Fig. 5C).
To examine whether phosphorylation of Thr$^{244}$ affects the subcellular distribution of APRIL, we first transfected COS cells with APRIL wt-FLAG or APRIL T244A-FLAG expression vectors. At 48 h post-transfection, the cellular and nuclear components of the respective transfected cell cultures were separated and subjected to Western blot analysis (Fig. 6A). Detection of the FLAG-tagged proteins revealed that wild-type APRIL is almost evenly distributed between the nucleus and cytoplasm (Fig. 6A, lanes 1 and 2). A completely different result was obtained when the intracellular distribution of the APRIL T244A mutant protein was examined, which was characterized by predominant nuclear localization (Fig. 6A, lanes 3 and 4).

These data suggested that restriction of APRIL to the cytoplasmic compartment depends on phosphorylation of Thr$^{244}$. Therefore we next analyzed the subcellular localization of endogenous APRIL in non-activated and PMA/ionomycin-stimulated Jurkat T cells. Strikingly, the Thr$^{244}$-phosphorylated protein was detected almost exclusively in the cytoplasm, irrespective of the fact whether the cells were activated or not (Fig. 6B, lanes 2 and 4 versus lanes 1 and 3, respectively). In contrast, total cellular APRIL was detected in both, the nucleus and the cytoplasm, although it appeared that slightly higher protein levels were present in the cytoplasm, particularly in activated T cells (Fig. 6B, lanes 3 versus lane 4). In contrast, the APRIL binding partner HuR localized mainly in the nucleus, and only comparably small amounts were detected in the cytoplasm upon T cell activation (Fig. 6B, lane 2 versus lane 4). Taken together, the data raised in Jurkat T cells confirmed the notion that the localization of APRIL to the nuclear or cytoplasmic compartment is determined by the phosphorylation status of Thr$^{244}$.

Phosphorylation of Thr$^{244}$ could lead to cytoplasmic retention of APRIL, for example by masking its NLS. Alternatively, phosphorylation may be necessary for nuclear export of the protein. To test this, we analyzed FLAG-tagged APRIL wt and T244A mutant protein with respect to their nucleocytoplasmic shuttling capacity by heterokaryon assay. Again, inclusion of GFP-Rev protein served as shuttling-positive internal control in these experiments (Fig. 6C, panels b and e). Inspection of the data revealed clearly distinct phenotypes. Whereas the wild-type protein exhibited shuttling behavior as before, appearing in both the human and mouse nuclei of heterokaryons, the phosphorylation-deficient mutant APRIL T244A was characterized by a severe nuclear export block, which was documented by its efficient accumulation in human nuclei and absence from mouse nuclei (Fig. 6C, panel c versus panel f). Thus, phosphorylation of Thr$^{244}$ in APRIL appears to be a prerequisite for translocation of this protein from the nucleus to the cytoplasm.

Because our data provided evidence that APRIL is required for efficient CD83 expression we next wanted to analyze the effect of the T244A (and NES3) mutant on CD83 expression. Clearly, overexpression of wild-type APRIL resulted in increased CD83 de novo synthesis (Fig. 6D). This effect was clearly less pronounced when the phosphorylation-deficient APRIL mutant T244A was overexpressed (lane 6 versus lane 4). Moreover, the nuclear export-deficient APRIL NES3 mutant displayed an inhibitory phenotype on CD83 expression when expressed in trans (lane 3 versus lane 1).

In sum, these data suggest that phosphorylation of Thr$^{244}$ triggers the translocation of APRIL from the nucleus to the cytoplasm and support the notion that phosphorylation of this residue in APRIL, as well as the APRIL NES, is required for CD83 expression.

**DISCUSSION**

The CD83 protein is considered to fulfill an important functional role in the interaction of immune cells (reviewed in Ref. 10). CD83 is a membrane glycoprotein expressed on the surface of activated T and B cells and, to a higher extent on fully mature
Moreover, circulating soluble CD83 protein is found at low levels in the serum of healthy individuals while, in contrast, significantly elevated levels are observed in patients suffering from certain hematological malignancies, such as lymphocytic leukemia and mantle cell lymphoma, or from rheumatoid arthritis (37–39). Interestingly, soluble CD83 efficiently inhibits DC-mediated T cell activation (11, 12, 40). Thus, CD83 appears to play a pivotal role in the immune modulation of T cell-mediated immune responses (8, 9).

It has been recently shown that the transcript encoding CD83 belongs to a small subset of cellular mRNAs that can exit the nucleus via an uncommon route. In general, the bulk of cellular mRNA is translocated from the nucleus to the cytoplasm by the transport factor TAP/NXF1 and its associated components (13–15). A recent systematic analysis of mRNA transport in T cells, however, revealed that a small number of inducible cellular mRNA species can be transported across the nuclear envelope by CRM1 (16), an export receptor that is usually exploited by U snRNA, ribosomal subunits, and various proteins (reviewed in Ref. 41). This finding is in agreement with previous independent studies showing that the transcripts encoding, for example, interferon-α, c-Fos, and cyclooxygenase-2 are, like the CD83 mRNA, subject to CRM1 regulation (42–44).

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FIGURE 5. Analysis of putative phosphorylation sites in APRIL and generation of phosphospecific anti-Thr244 antiserum. A, putative phosphorylation sites in human APRIL (ANP32B) were predicted by in silico calculation (open boxes). Phosphorylation of Thr244 was confirmed by MALDI-TOF mass spectrometry (not shown) and is indicated by a filled box. Amino acid residues constituting the basic NLS (amino acids 239–242) and the leucine-rich NES (amino acids 109–120) of APRIL are underlined. B, a Thr244-phosphorylated APRIL peptide was used to generate phosphospecific anti-pT244 antiserum in rabbits. GST-APRIL fusion protein purified from E. coli (lanes 2, 4, and 6) and B cell-derived native APRIL (lanes 1, 3, and 5) was analyzed. Corresponding Western blots were probed with anti-APRIL (lanes 1 and 2), nonpurified anti-pT244 (lanes 3 and 4), and affinity-purified anti-pT244 (lanes 5 and 6) antiserum, respectively. C, the phosphospecific anti-pT244 antiserum fails to recognize an APRIL mutant, in which Thr244 was substituted by Ala (APRIL T244A). COS cells were either transfected with the parental expression vector (lane 1, Control) or transfected with vectors encoding FLAG-tagged versions of APRIL wt (lane 2) or APRIL T244A (lane 3) protein. Western blot analyses of the respective cell extracts were performed by using antibodies directed against α-tubulin (anti-α-tubulin; gel loading control), the FLAG epitope (anti-FLAG), APRIL (anti-APRIL), and Thr244-phosphorylated APRIL (anti-pT244).
Taken together, these findings provided ample evidence that the HuR-CRM1 axis is operational in the nuclear export of some cellular mRNAs, particularly in the intracellular transport of the message encoding CD83. Because HuR cannot directly interact with the export receptor CRM1, the HuR ligands pp32 and APRIL have been suggested to act as link that provide access of HuR to the CRM1 nuclear export pathway (19, 20). By identifying the phosphoprotein APRIL to be an important player in the nucleocytoplasmic translocation of CD83 mRNA, we provide another component of the complex ribonucleoprotein (RNP) framework that is involved in the posttranscriptional regulation of CD83 expression.

Both, pp32 and APRIL belong to the growing family of ANP32 proteins (ANP32a-h), a group of leucine-rich acidic nuclear phosphoproteins, which have been linked to manifold activities in conjunction with the regulation of gene expression, cell signaling, cytoskeleton dynamics, neural development, cell adhesion, and cell death (reviewed in Ref. 36). By using RNAi technology, we were able to demonstrate that the two prototypical ANP32 family members, pp32 (ANP32A) and APRIL (ANP32B), can be differentiated with respect to their impact on CD83 expression. In sharp contrast to pp32 knock down, the silencing of APRIL resulted in significantly decreased cytoplasmic levels of CD83 mRNA. Obviously, other ANP32 proteins, such as pp32, cannot substitute for APRIL activity in this context. This finding was surprising, since in previous studies pp32 and APRIL could not be dissected with respect to HuR-mediated ARE RNA transport (20, 43). It therefore appears that the RNP composition required for HuR-mediated nuclear export of CD83 mRNA is, with respect to the protein ligands of HuR, different from the composition required for a typical ARE-containing transcript. This notion is in agreement with current models that suggest that the individual RNP composition on a given mRNA determines the posttranscriptional fate of this transcript with respect to its nuclear export, stability, and indirectly translation (46, 47).

The finding that pp32 and APRIL execute different activities is not without precedence. For example, it has already been earlier reported that pp32, but not APRIL, is a potent inhibitor of the heat-stable protein phosphatase 2A (PP2A), the major mammalian protein serine-threonine phosphatase (36, 48). Our further analysis of pp32 and APRIL also demonstrated that endogenous APRIL is more abundant than pp32 in CD83-expressing Jurkat T cells, which can be explained by APRIL’s sig-

FIGURE 6. Phosphorylation of Thr244 affects the subcellular distribution of APRIL. A, COS cells were transfected with expression vectors encoding APRIL wt-FLAG (lanes 1 and 2) or APRIL T244A-FLAG (lanes 3 and 4). Two days post-transfection, cytoplasmic (C) and nuclear (N) proteins were prepared and subjected to Western blot analysis using α-tubulin-, FLAG-, HuR-, or hnRNA A1-specific antibodies. In addition, the blotting membrane was stained with Ponceau reagent. Detection of HuR and hnRNP A1 served as marker of the nuclear fraction. B, nuclear (N) and cytoplasmic (C) protein extracts of non-activated (−) and PMA/ionomycin-activated (+) Jurkat T cells were prepared and analyzed as before using phosphospecific anti-pT244, anti-APRIL, anti-HuR, anti-hnRNP A1, and anti-α-tubulin antibodies or Ponceau staining reagent. C, HeLa cells were cotransfected with p3UTR-CD83 and expression vectors encoding GFP-Rev (shuttling-positive control) and either APRIL wt-FLAG (corresponding panels a–c) or APRIL T244A-FLAG (corresponding panels d–f). Heterokaryon analysis was performed as described above. The cells were stained with Hoechst 33258 (panels a and d) and anti-FLAG antibody (panels c and f). GFP-Rev protein was detected by exploiting its intrinsic fluorescence (panels b and e). Human nuclei are indicated by arrows. Corresponding images are arranged on top of each other. D, phenotype of APRIL mutants on CD83 expression in trans. COS cells were cotransfected with p3UTR-CD83 and expression vectors encoding the indicated APRIL proteins. At day 3 post-transfection, cell cultures were radiolabeled, and the respective cellular extracts were subjected to CD83-specific immunoprecipitation, followed by SDS-PAGE. CD83 protein was detected by autoradiography, and the obtained signals were quantified by phosphorimage analysis (indicated in percent). In addition, each radiolabeled extract was controlled by α-tubulin-specific Western blot analysis.
significant longer half-life of ~28 h, as compared with the pp32 half-life of ~12.5 h. This finding was also unexpected, because both proteins exhibit striking structural similarity of ~71% sequence identity and over 81% sequence homology (19). Thus, although being members of the same family of proteins both, pp32 and APRIL, appear to exert distinct functions with respect to various aspects of cellular regulation.

The detailed analysis of the signals that direct the shuttling of APRIL resulted in the mapping of four basic amino acids in the C terminus of human APRIL (NH$_2$-KRKR-COOH at amino acid positions 239–242) that serve as the protein NLS. This observation confirms an earlier report that this sequence is part of the NLS of murine ANP32e (mLANP-L) and may therefore constitute the import signal in the majority of the ANP32 family of proteins (49). In addition, by identifying the NES in APRIL (NH$_2$-LKKLECLKSLDL-COOH at amino acid position 109–120), which appears to be a prototypic leucine-rich repeat (50), we functionally determined the interface for interaction of APRIL with the export receptor CRM1.

APRIL is, like many other ANP32 proteins, potentially modified by phosphorylation (36). However, only a limited number of phosphorylation sites have yet been determined in this protein. In the present study we were able to identify threonine 244 in human APRIL to be partially phosphorylated. Threonine 244 is of particular interest because this residue is located in close vicinity of the NLS and may therefore, as shown recently in case of the CDK inhibitor p27 (51), interfere with binding to importin α. In agreement with this notion, our analysis demonstrated that Thr$_{244}$-phosphorylated APRIL resides exclusively in the cytoplasm. However, further analyses revealed that the unphosphorylated Thr$_{244}$ version of APRIL localizes in the cell nucleus and that Thr$_{244}$ has to be phosphorylated before nuclear export of APRIL occurs. It therefore appears that the functional interaction of APRIL with CRM1 is directly or indirectly regulated by nuclear phosphorylation of APRIL. Examples for this type of CRM1-specific regulation of nuclear export has been described for various cellular proteins, including the prereplicative complex Mcm2-7 in yeast (52), the CDK inhibitor p27 (53), and cyclin D1 (54). Clearly, the presented findings will allow future studies to elucidate APRIL activity with respect to CD83 expression in more detail. Particularly the identification of the Thr$_{244}$-phosphorylating kinase and the subsequent functional analysis of this APRIL-modifying enzyme is expected to provide novel insights into the post-transcriptional processing of CD83 mRNA during T cell activation and DC maturation. Furthermore, the inhibition (e.g. by RNAi) of this kinase may extend our so far limited knowledge of the HuR role in the regulation of primary immune responses.

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