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Stimulated Expression of mRNAs in Activated T Cells Depends on a Functional CRM1 Nuclear Export Pathway

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In metazoans, the nuclear export of bulk mRNAs is mediated by the export receptor TAP, together with its binding partner p15. A number of viral mRNAs, including the unspliced and partially spliced mRNA species of the human immunodeficiency virus (HIV), however, use an alternative export route *via* the importin β-related export receptor CRM1. This raises the question of whether a subset of cellular mRNAs might be exported by CRM1 as well. To identify such mRNAs, we performed a systematic screen in different cell lines, using representational difference analyses of cDNA (cDNA-RDA). In HeLa and Cl-4 cells no cellular transcripts could be identified as exported *via* CRM1. In contrast, we found a number of CRM1-dependent mRNAs in Jurkat T cells, most of which are induced during a T cell response. One of the identified gene products, the dendritic cell marker CD83, was analyzed in detail. CD83 expression depends on a functional CRM1 pathway in activated Jurkat T cells as well as in a heterologous expression system, independent of activation. Our results point to an important role of the CRM1-dependent export pathway for the expression of CD83 and other genes under conditions of T cell activation.

Keywords: RNA export; CRM1; T cell activation; representational difference analysis; CD83

Introduction

Nuclear export of different classes of RNA is mediated by different factors.[1] In recent years, transport pathways and transport factors for all classes of RNA have been identified. RNAs are always transported as RNA–protein complexes. Hence, it is not surprising that RNA transport is governed by similar principles as nuclear protein transport.[2–4] Indeed, most of the transport receptors involved in nuclear RNA export belong to the well-characterized family of importin β-like proteins, also referred to as karyopherins, which mediate nuclear transport of proteins in both directions. Importin β, the prototype of this family, is the transport receptor for nuclear import of proteins containing a "classic" nuclear localization signal. Examples for importin β-like proteins involved in RNA transport are the export receptors exportin-t for tRNA,[5,6] exportin-5 for microRNA precursors[7,8] and CRM1 for U snRNAs[9,10] and ribosomal subunits.[11,12]

mRNA appears to be the only major species of RNA that takes an export route independent of importin β-like proteins.[13] The transport factors TAP/NXF1 and p15/NXT1 are required for export of the bulk of cellular mRNA,[14,15] and their depletion or inactivation leads to nuclear accumulation of poly(A)$^+$ mRNA.[16–19] Additional factors are involved in the formation of export complexes, as TAP and p15 themselves do not bind to cellular mRNAs. A picture is emerging where mRNA export is tightly coupled to mRNA transcription, splicing, and also 3′ end formation (for a review, see Reed & Hurt[20]).

CRM1 is the major cellular transport receptor for export of proteins out of the nucleus.[10,21–24] CRM1

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Abbreviations used: HIV, human immunodeficiency virus; LMB, leptomycin B; NES, nuclear export signal; RDA, representational difference analysis; GFP, green fluorescent protein.

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interacts with so-called leucine-rich nuclear export signals (NESs), which were originally identified in the inhibitor of the cAMP-dependent protein kinase (PKI[25]) and the Rev-protein of the human immunodeficiency virus (HIV[26]). HIV-Rev serves as an adapter protein for CRM1-dependent export of unspliced and partially spliced viral mRNAs. This raises the question of whether CRM1 may also function in cellular mRNA export. Indeed, some cellular transcripts have been identified that seem to use CRM1 as an export receptor. These include the transcripts for interferon-α1,[27] the proto-oncogene c-fos,[28] and the cyclooxygenase COX-2.[29] Interestingly, the TAP-related protein NXF3, which is mainly expressed in testis, contains a leucine-rich NES which mediates its interaction with CRM1 and has the ability to export mRNA.[30] Hence, NXF3 may serve as a tissue-specific adapter protein for CRM1-dependent transport of specific mRNA molecules.

Here, we used cDNA representational difference analysis (cDNA-RDA)[31] to systematically search for cellular RNA species that are exported *via* the CRM1-pathway. This method is based on the subtractive hybridization of two cDNA populations and subsequent amplification of sequences enriched in one of the two populations. In stimulated T cells, we identified a number of mRNAs whose cytoplasmic concentration is strongly reduced in the presence of the CRM1-inhibitor leptomycin B (LMB) or upon transfection of cells with a fragment of the nucleoporin Nup214 (CANc), which is known to inhibit CRM1-dependent transport. For one of the identified clones, the dendritic cell marker CD83, we observed a specific reduction in the expression level in a heterologous expression system, when the CRM1 pathway was inhibited. Our results point to an important role of the nuclear export receptor CRM1 in the expression of a subset of genes under conditions of cellular activation or differentiation.

Results

It has been suggested that a certain percentage of cellular mRNA molecules are exported *via* the CRM1-pathway.[32] Here, we devised a screen that allows the identification of such mRNA species. RNA molecules exported out of the nucleus in a CRM1-dependent fashion will be depleted from the cytoplasm upon inhibition of the CRM1 pathway. We used LMB for inhibition of CRM1-mediated nuclear export. LMB is a fungal metabolite that covalently modifies CRM1,[33] thereby inhibiting CRM1-dependent export.[10,34] Inhibition by LMB is highly specific, as CRM1 is its only cellular target.[33]

In our experimental approach, we compared either cytoplasmic or nuclear RNA populations of cells treated with or without LMB. We used cDNA-RDA for the comparison, a PCR-based method that has been used for the analysis of differential gene expression in various tissues and cell lines.[31] Briefly, cDNA derived from RNA from two populations is cut with the endonuclease DpnII and ligated to linker oligonucleotides. After PCR amplification, the resulting representations (i.e. cDNAs representing the original mRNA pool) are again digested with DpnII to remove the original oligonucleotide. A second oligonucleotide is then ligated to the representation that is supposed to contain the sequences of interest at higher concentrations (the "*tester*"). The *tester* is then hybridized with an excess of the other representation (the competitor or "*driver*"), which lacks the new oligonucleotide ends. Subsequent amplification with the second oligonucleotide as primer results in exponential enrichment of *tester-tester* hybrids, leading to difference product 1 (DP1). The procedure of exchanging the linker oligonucleotide in the difference products, hybridization to increasing amounts of *driver* and PCR amplification is repeated to obtain DP2, DP3 etc. With this procedure, sequences are obtained that are enriched in the *tester* representation and therefore also in the original RNA population. These sequences are then cloned into an appropriate vector for further analysis.

Validation of the method: isolation of HIV-env and rRNA sequences

To demonstrate the validity of our screening method, we set out to identify an mRNA species known to be exported by CRM1. Cl-4 cells expressing the HIV-env mRNA in an HIV-Rev (i.e. CRM1)-dependent fashion,[35] were incubated with or without LMB for 24 h and nuclear and cytoplasmic fractions were prepared. The quality of the subcellular fractionation was assessed by Northern blotting, using a probe that detects U6 snRNA, an RNA species that is expected not to leave the nucleus[36] (data not shown). For experiments using nuclear RNA as starting material, plus-LMB representations were used as *tester*, as these were expected to contain sequences that are enriched in the nucleus upon CRM1-inhibition. For experiments using cytoplasmic RNA, the plus-LMB representations were now used as *driver*, as CRM1-dependent RNAs should be depleted from the cytoplasm in the presence of the drug.

Figure 1 shows a typical example of the first difference products (DP1–DP3), obtained after one, two or three rounds of subtractive hybridization and subsequent amplification. Cytoplasmic RNA from Cl-4 cells incubated with or without LMB was used as starting material. DP1 usually consists of a complex smear,[37] whereas distinct bands in DP2 and DP3 may correspond to specific cDNAs that are amplified during the procedure. We cloned the entire difference products DP2 and DP3 into a plasmid vector. From each experiment about 400 colonies were transferred to membranes for further screening analyses and selected clones were sequenced.

The CRM1-dependent HIV-env sequence expressed in Cl-4 cells was detected in ~1% of the clones from an RDA with cytoplasmic Cl-4

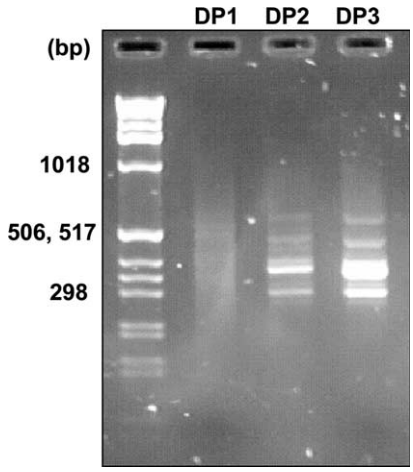

Figure 1. Enrichment of difference products (DP) in cytoplasmic RDA after treatment of Cl-4 cells with or without LMB: 1/40 of difference products DP1–DP3 were resolved by agarose gel electrophoresis and visualized by UV light.

RNA. β-Actin and GAPDH-probes did not hybridize to the colonies although these sequences were still present in the representations (see Figure 2(c)). This result indicates that very abundant sequences that are not present at different levels in the original mRNA pool were lost during the RDA.

To characterize the behavior of HIV-env sequences upon treatment of cells with LMB, we performed Northern blot hybridizations using cytoplasmic and nuclear RNA from LMB-treated or untreated cells. As shown in Figure 2(a), HIV-env RNA was depleted from the cytoplasmic RNA pool upon LMB-treatment, whereas no difference was observed in the nuclear levels of the message. As the cytoplasmic signal for the HIV-env RNA was rather low, we performed quantitative RT-PCR. This analysis revealed a fivefold higher level in cytoplasmic HIV-env RNA in untreated *versus* LMB-treated cells (Figure 2(b)). The nuclear level of env-RNA, which was two to threefold higher in untreated cells compared to the corresponding cytoplasmic level (data not shown), did not change upon LMB-treatment (Figure 2(b)), in agreement with the results presented in Figure 2(a). In contrast to HIV-env, the level of cytoplasmic β-actin-mRNA was not affected by LMB (data not shown).

We also analyzed the original representations (i.e. cDNAs after reverse transcription and DpnII digestion) with an HIV-env probe. As shown in Figure 2(c), one of the expected DpnII fragments derived from the HIV-env sequence is strongly depleted in the representation of cytoplasmic RNA derived from cells treated with LMB (the *driver*). In contrast, similar levels of β-actin sequences could be detected in both representations, suggesting that the corresponding transcript is not affected by treatment of cells with LMB. As a consequence, β-actin cDNA-fragments are not amplified by RDA.

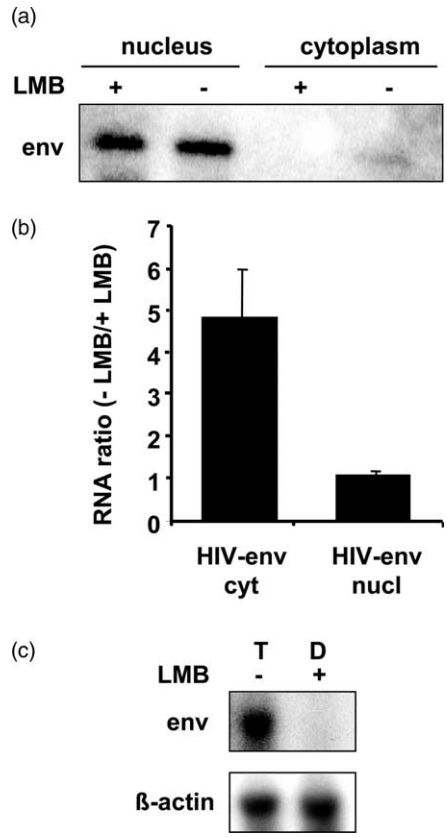

Figure 2. CRM1-dependent HIV-env RNA can be identified by RDA. (a) Northern blot with nuclear and cytoplasmic poly(A)$^+$ RNA from Cl-4 cells treated with or without LMB, detecting HIV-env sequences. (b) Quantitative RT-PCR detecting HIV-env sequences. Data were expressed as cytoplasmic (cyt) or nuclear (nucl) ratios of RNA in cells treated without or with LMB (−LMB/+LMB). The mean of two (nucl) or three (cyt) quantifications is shown. (c) cDNA Southern blot of cytoplasmic representations from Cl-4 cells probed with an HIV-env-specific DpnII-fragment as isolated by RDA, and a human β-actin probe as control. T, *tester* representation; D, *driver* representation.

Note that the comparison of cDNA representations by Southern blotting is a qualitative analysis. As a result of many PCR-amplification steps, control cDNAs (β-actin, GAPDH; see Figures 2(c), 3(a) and 4) may vary in abundance to some extent. After the initial screening experiments, identified clones must therefore be analyzed at the level of the mRNA. These results show that the differences in relative abundance as detected by Northern blotting or RT-PCR are retained and even enhanced at the level of the cDNA representations. The high concentration of HIV-env sequences in the *tester* compared to the *driver* allowed their amplification during the RDA, validating our experimental approach for the identification of CRM1-dependent mRNAs.

In nuclear RDAs from Cl-4 cells and HeLa cells, a large percentage of isolated clones corresponded to sequences derived from 18 S or 28 S rRNA. Given the high abundance of rRNA sequences in our

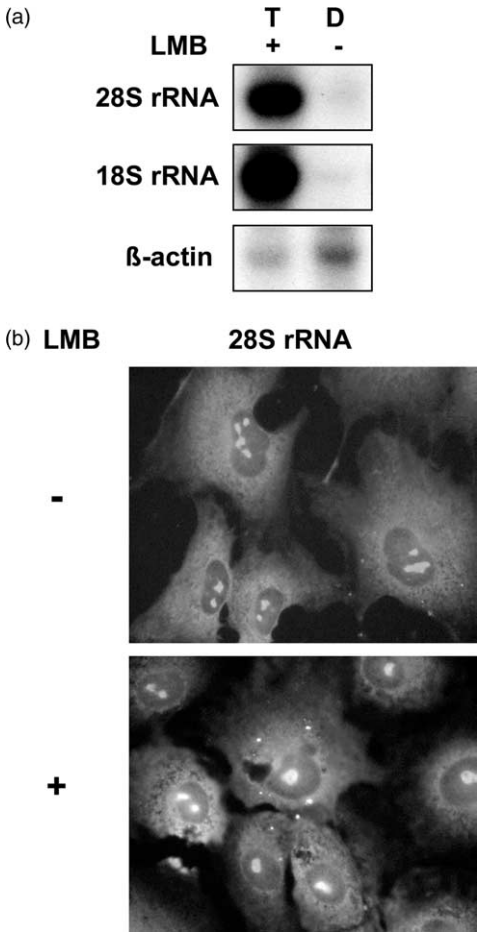

Figure 3. CRM1-dependent export of ribosomal RNAs. (a) cDNA Southern blot of nuclear representations probed with DpnII-fragments of 18 S rRNA, 28 S rRNA or β-actin sequences, as indicated. T, *tester* representation; D, *driver* representation. (b) Fluorescence *in situ* hybridization detecting 28 S rRNA. (a) and (b) Cells were treated with (+) or without (−) 15 nM LMB overnight.

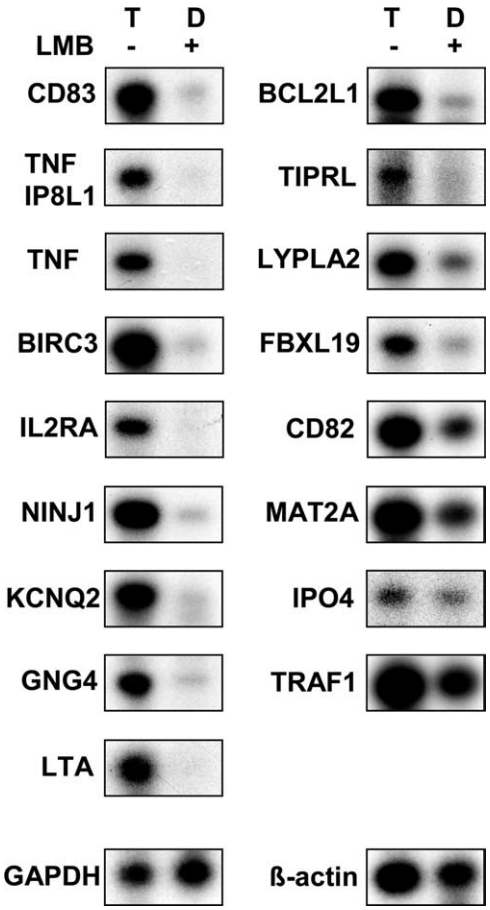

Figure 4. cDNA Southern blot with 5 μg of cytoplasmic representations from activated Jurkat T cells. Cells were treated with (+) or without (−) 20 nM LMB for 3.5 h. DpnII fragments of the indicated clones were used as radioactive probes, β-actin and GAPDH PCR product probes were used as controls. T, *tester* representation; D, *driver* representation.

RDAs, we asked whether their amplification might be of biological significance. Indeed, in yeast cells, the nuclear export of the large ribosomal subunit has been shown to occur *via* the CRM1 pathway.[38,39] When cDNA representations were hybridized with rRNA-specific probes, a strong accumulation of both 18 S and 28 S sequences was observed in the nuclear representation derived from cells incubated with LMB (the *tester*; Figure 3(a)). To test whether the inhibition of the CRM1-pathway affects the subcellular localization of rRNA in our cells, we compared the distribution of 28 S rRNA in Cl-4 cells treated with or without LMB by fluorescence *in situ* hybridization. As shown in Figure 3(b), 28 S rRNA was detected in nucleoli as well as in the cytoplasm of control cells. Upon incubation of cells in the presence of LMB, 28 S rRNA accumulated in nucleoli. The nucleolar to cytoplasmic ratio of fluorescence intensities increased from 2.5 ± 0.6 in the absence of LMB to 4.3 ± 0.7 in the presence of LMB ($n = 28$). These results are in agreement with previously published observations,[11,12] describing the CRM1-dependent nuclear export of the large ribosomal subunit in mammalian cells. Thus, the isolation of several rRNA fragments by RDA is likely to result from this CRM1-dependent transport pathway and serves as a further proof of principle for the feasibility of RDA for the identification of CRM1-dependent transcripts.

Isolation of CRM1-dependent mRNAs from activated T cells

Nuclear and cytoplasmic RDAs using Cl-4 cells or HeLa cells yielded a number of clones derived from mRNA sequences. To further characterize these transcripts, we performed Southern blotting of representations, using cDNA derived from cells that had been treated with or without LMB for four, 13, or 24 h. Large differences between these representations, as observed for the HIV-env sequence (Figure 2(c)), however, could not be detected for these clones (data not shown). Likewise, no major differences for the putative CRM1-dependent mRNAs could be detected by Northern

blotting or RT-PCR (data not shown). We conclude from these results that the CRM1-dependent transport pathway does not play a significant role in nuclear export of constitutively expressed cellular mRNAs in mammalian cells, in agreement with similar results from insect cells.[40]

The few cellular CRM1-dependent mRNAs described so far are not constitutively expressed, but rather induced upon cellular stimulation or differentiation. We therefore performed RDA experiments with Jurkat T cells, a human T cell line, under conditions that simulate a T cell response. T cells are also a major host for HIV, where CRM1-dependent RNA-export is well documented. Activated Jurkat cells were treated with or without LMB for 3.5 h and RDA was carried out with cytoplasmic mRNA as starting material. After three rounds of subtractive hybridization, single clones were isolated and sequenced. Analysis of 52 clones revealed 18 different cDNAs (Table 1). When we compared the signal intensities on cDNA Southern blots, clear differences between the *tester* (i.e. the cytoplasmic cDNA population from cells treated without LMB) and the *driver* were observed for the majority of the clones (Figure 4; see also Table 1). The levels of β-actin and GAPDH were very similar in the two representations.

We next analyzed the effect of LMB on the putative CRM1-dependent sequences at the mRNA-level. Many of the candidate mRNAs could be detected by Northern blotting (Figure 5(a)), whereas others required semi-quantitative RT-PCR as a more sensitive method for detection (Figure 5(b)). These experiments revealed that the analyzed transcripts are induced upon T cell activation (see Supplementary Data, Figure S1), and also show strongly reduced cytoplasmic levels upon treatment of cells with LMB. These results explain the selective amplification of the corresponding cDNAs by RDA. The nuclear levels of most transcripts were also significantly reduced in LMB-treated cells, suggesting changes in transcription and/or degradation rates of sequences that are sequestered in the nucleus. Enhanced degradation of nuclear RNA upon inhibition of nuclear export has been observed previously.[40–42] We also performed quantitative RT-PCR for one of our identified sequences, the mRNA coding for CD83. As a control, we used an mRNA of similar abundance (i.e. much lower than β-actin), the one coding for the alpha-subunit of the trimeric G-protein Gαs. As shown in Figure 5(c), ~fivefold higher levels of CD83 mRNA were detected in nuclear and cytoplasmic fractions of cells that had not been treated with LMB, compared to treated cells. These results are in good agreement with those shown in Figure 5(a). In contrast, levels of the Gαs-message did not change significantly upon LMB-treatment, suggesting that the drug affects only a subset of mRNAs.

To further verify the CRM1-dependence of the identified transcripts, we performed another analysis, independent of LMB. A fragment of the nucleoporin Nup214 has been shown to inhibit the CRM1-dependent export pathway when transfected into cells.[43,44] TAP-mediated mRNA export, on the other hand, is not affected by this fragment.[44] We co-transfected an excess of a plasmid coding for a C-terminal fragment of Nup214 (CANc) together with a plasmid coding for the green fluorescent protein (GFP) into Jurkat cells and stimulated them to express the mRNAs of interest. GFP-positive cells were sorted by flow cytometry and total, nuclear and cytoplasmic RNA levels of candidate transcripts were analyzed by semi-quantitative RT-PCR. We also included mRNAs coding for c-fos, which had previously been suggested to be exported *via* CRM1, as well as GM-CSF and interleukin 2 (IL-2)

Table 1. cDNA-RDA clones from activated Jurkat T cells

Gene	Name	Acc.no.	Fold change	Activation
Tumor necrosis factor α-induced protein 8-like	1TNFAIP8L1	BC044250	30.5	n.d.
Tumor necrosis factor α	TNFα	NM_000594	25.3	+
Baculoviral IAP repeat containing 3	BIRC3	NM_182962	8.2	+
Interleukin 2 receptor α	IL2RA	K03122	6.4	+
Ninjurin 1	NINJ1	BC004440	6.25	+
CD83 antigen	CD83	NM_004233	6.1	+
Potassium voltage-gated channel	KCNQ2	BC000699	4.3	n.d.
Guanine nucleotide binding protein γ 4	GNG4	NM_004485	4.2	+
Lymphotoxin α	LTA	NM_000595	3.6	+
BCL2-like 1	BCL2L1	BC019307	2.6	+
Full-length cDNA clone	CS0D1039YL07	CR617576	2.5	n.d.
Putative MAPK activating protein	TIPRL	AB097034	2.1	n.d.
Lysophospholipase II	LYPLA2	NM_007260	1.9	n.d.
F-Box & leucine-rich repreat protein 19	FBXL 19	NM_019085	1.8	n.d.
CD82 antigen	CD82	NM_002231	1.8	+
Methionine adenosyltransferase II α	MAT2A	BC001854	1.7	+/−
Importin 4	IPO4	BC003690	1.6	n.d.
TNF receptor associated factor I	TRAF1	BC024145	1.4	+

Fold change: fold signal intensity of the *tester* representation as compared to the *driver* representation, as revealed by cDNA Southern blotting. Activation: induction of transcript by PMA/ionomycin treatment as seen by Northern blotting or RT-PCR. n.d., not determined.

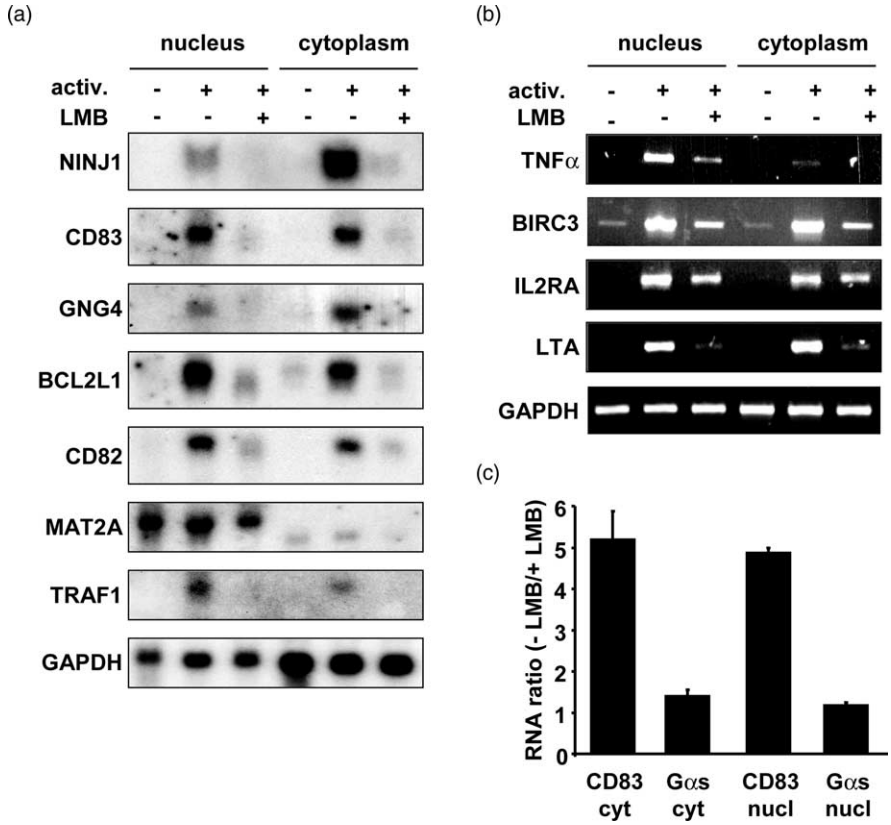

Figure 5. mRNAs found by RDA are inducible by PMA and ionomycin and decrease in levels upon treatment of Jurkat cells with LMB. (a) Northern blot of nuclear and cytoplasmic RNA fractions from untreated, activated and activated/LMB-treated cells. DpnII fragments of the indicated RDA clones and a GAPDH-PCR fragment were used as radioactive probes. (b) Semi-quantitative RT-PCR using equal amounts of RNA from nuclear and cytoplasmic RNA fractions from untreated (activ. −), activated (activ. +), and activated and LMB treated cells, detecting various transcripts. (c) Quantitative RT-PCR using cytoplasmic (cyt) or nuclear (nucl) RNA from activated Jurkat cells, detecting the CD83 or the Gαs-message. Data (mean of two quantifications) were expressed as ratios of RNA in cells treated without or with LMB (−LMB/+LMB) for 3.5 h.

in our analysis. In Jurkat cells, these mRNAs are also induced upon activation (see Supplementary Data, Figure S1). However, the corresponding cDNAs do not contain appropriate DpnII restriction sites, precluding their detection by RDA. Nevertheless, treatment of cells with LMB leads to a clear reduction of the cytoplasmic concentration of c-fos, GM-CSF and IL-2 mRNAs (data not shown). As shown in Figure 6, the mRNAs can be grouped into three classes: (i) for most transcripts (S14, coding for a ribosomal protein as a control, BIRC3, Il2RA, LTA, BCL2L1, c-fos, NINJ1), transfection of the inhibitory fragment of Nup214 did not result in a proportionally higher RNA-level in the nucleus, compared to the cytoplasm. mRNAs coding for BCL2L1 and c-fos were present at significant levels prior to induction (T^-), suggesting that their expression is already triggered by the transfection (compare Supplementary Data, Figure S1). The observation that the distribution of many mRNAs of various abundances is not affected by the transfection of the Nup214-fragment serves as a specificity control for the effects on the following RNAs. (ii) For three RNAs (GNG4, TNFα, CD83), the Nup214-fragment slightly increased the nuclear level of the transcripts, compared to their cytoplasmic level. (iii) For two mRNAs (IL-2 and GM-CSF), this effect was strongly enhanced. These results further support the notion of CRM1-dependent expression of a subset of mRNAs during T cell activation.

To avoid potential problems with side-effects on T cell activation *per se* under conditions of CRM1 inhibition, we analyzed the expression of a candidate protein in a heterologous system. We chose CD83 for this analysis, as its mRNA was affected by LMB as well as by the inhibitory Nup214-fragment. 293T cells were co-transfected with plasmids coding for CD83 and GFP and an excess of either pcDNA3-CANc, coding for the inhibitory Nup214-fragment, or the empty vector pcDNA3. Three days after transfection, cells were harvested and analyzed for the expression of GFP and CD83 by flow cytometry. As shown in Figure 7(a), the co-expression of the Nup214-fragment reduced the normalized expression level of CD83 to about 60% of the control level. As expected, it had only a minimal effect on GFP-expression (which was driven from a CMV-promotor, like CD83; data not shown). Cells that were positive for CD83 or GFP were also positive for the Nup214-fragment, indicating an

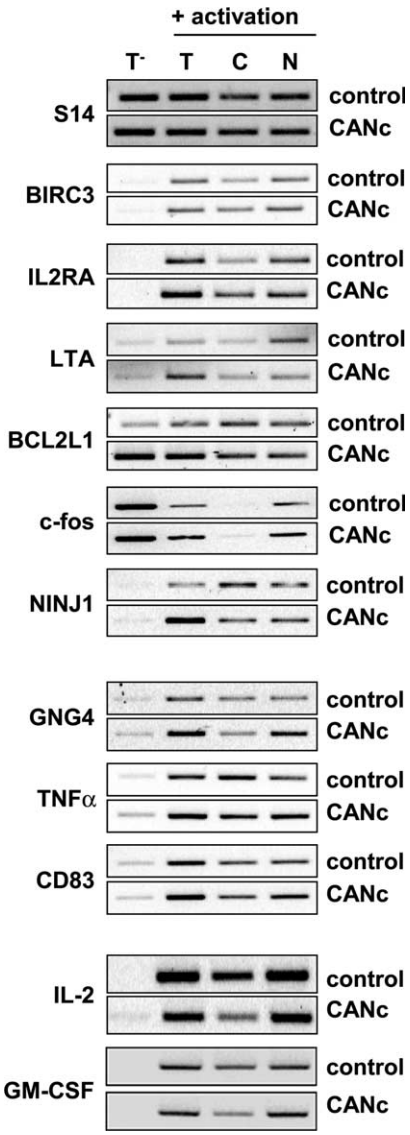

Figure 6. Inhibition of CRM1 by an inhibitory Nup214-fragment leads to a reduced cytoplasmic concentration of selected mRNAs. Jurkat cells were co-transfected with a plasmid coding for the C terminus of Nup214 (CANc) or the empty vector (control), together with a plasmid coding for GFP. Cells were activated to express mRNAs of interest and GFP-positive cells were sorted by FACS. Total RNA levels of untreated cells (T^-), as well as total (T), cytoplasmic (C) and nuclear (N) levels of activated cells were subjected to RT-PCR. The number of cycles was adjusted to the level of the individual messages, allowing a semi-quantitative analysis.

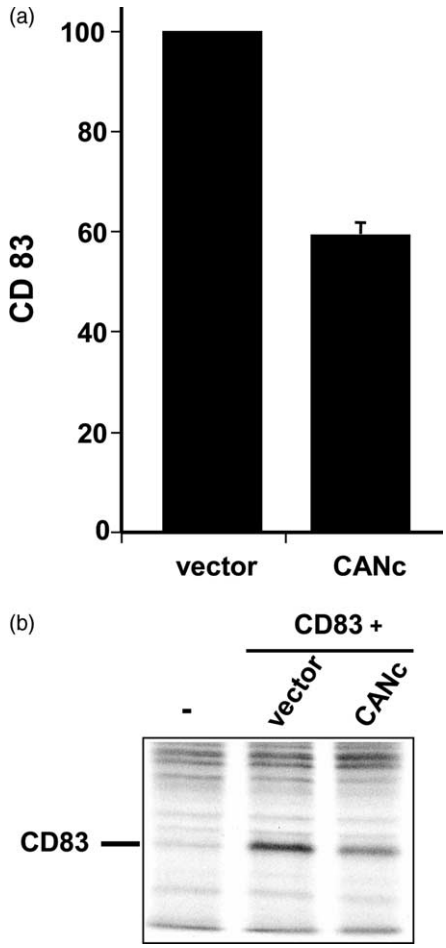

Figure 7. Expression of transiently transfected CD83 is reduced by the inhibitory Nup214 fragment. (a) 293T cells were cotransfected with plasmids coding for GFP, CD83 and either the C terminus of Nup214 (CANc) or the empty vector (vector). CD83-expression levels were measured by flow cytometry and normalized to GFP-levels. The mean of four separate experiments is shown. The bar indicates the standard deviation. (b) COS-7 cells were cotransfected with plasmids coding for CD83 and either the C terminus of Nup214 (CANc) or the empty vector (vector). Cells were metabolically labelled and lysates were subjected to immunoprecipitation with an antibody against CD83 and analyzed by SDS-PAGE. The background labelling serves as a loading control.

efficient cotransfection (data not shown). Under these conditions, a certain proportion of the detected CD83 molecules had probably been synthesized soon after transfection, when the Nup214-fragment had not yet accumulated to a level sufficient to inhibit CRM1. We therefore also analyzed the expression of CD83 by metabolic labelling of transfected cells, followed by immunoprecipitation. As shown in Figure 7(b), in cells cotransfected with the Nup214-fragment, the level of newly synthesized CD83 was reduced to about 50% compared to a control transfection. Taken together, the CRM1-dependence of CD83 does not depend on the cellular context of activated T cells but can be recapitulated in a heterologous expression system.

Discussion

CRM1-dependent RNAs can be detected by RDA

Here, we developed a screen to identify cellular mRNAs that are exported *via* the CRM1 pathway.

We were able to isolate two types of RNAs that are known to use this transport pathway, thereby validating our experimental approach. First, we isolated clones derived from the HIV-env mRNA, an established CRM1 target whose export is mediated by the viral NES-containing protein HIV-Rev.[45] HIV-env sequences were isolated by cytoplasmic RDA (i.e. cytoplasmic RNA was used as starting material) but not nuclear RDA (i.e. nuclear RNA was used as starting material). When nuclear export of the HIV mRNA coding for Env is inhibited by LMB, the transcript is subject to further splicing and/or degradation.[46] Therefore, we were unable to detect differences in nuclear levels of env-mRNA in LMB-treated *versus* untreated cells. The message is depleted from the cytoplasm, however, allowing efficient amplification during cytoplasmic RDA.

Second, a significant number of RDA-clones from our initial screens in HeLa and Cl-4 cells corresponded to 18 S and 28 S rRNAs. rRNA sequences are almost always present in cDNA preparations and may also be amplified in cDNA RDA experiments.[37] The amplification of rRNA sequences in our RDA clearly resulted from differences in concentration in the nuclear cDNA representations. Our *in situ* hybridization data further showed that LMB affects the subcellular distribution of 28 S rRNA under our experimental conditions, supporting the notion that the large ribosomal subunit is exported *via* the CRM1-pathway. rRNA is known to have a long half-life, so after inhibition of export, its nuclear accumulation is expected to be seen prior to its depletion from the cytoplasm. In yeast cells and in mammalian cells, Nmd3 has been identified as an adapter protein that mediates the interaction between the large ribosomal subunit and CRM1.[11,12,38,39] The adapter protein for export of the small ribosomal subunit remains to be identified. Taken together, RDA is a valid method for the identification of CRM1-dependent mRNAs.

Export of constitutively expressed mRNAs is not mediated by CRM1

Under RDA conditions that allowed the identification of HIV-env and rRNA sequences, no CRM1-dependent mRNAs were detected, either in HeLa cells or in CL-4 cells. We conclude from these results that the CRM1-dependent mRNA export pathway does not play a significant role in these cell lines. Our observations are in line with results described by Herold *et al.*,[40] who used microarray technologies to analyze the effect of CRM1 inhibition by LMB in insect cells. It remains possible that CRM1 supports export of certain mRNA–protein complexes, but is not absolutely required, as suggested for the Balbiani ring mRNP.[47] Likewise, we cannot exclude the possibility that the half-life of a particular CRM1-dependent mRNA is too long to detect significant concentration differences in our experimental setup upon inhibition of CRM1.

CRM1-dependent mRNAs in activated T cells

As the bulk of constitutively expressed mRNAs are clearly not exported *via* CRM1, we expanded our search to a well-characterized inducible model system. In activated Jurkat T cells, we identified a number of CRM1-dependent mRNAs (Table 1), which are not constitutively expressed, similar to the previously described CRM1 targets.[27–29] Besides the cytoplasmic depletion of these transcripts upon inhibition of the CRM1-export pathway, we observed a reduction in their nuclear levels. A similar observation was made by Herold *et al.*[40] after depleting the bulk mRNA export receptor TAP by RNAi in Drosophila cells. TAP-depleted cells showed a reduction in total RNA levels, suggesting a decreased RNA level in the cytoplasm as well as in the nucleus. This effect has also been observed in yeast cells where mRNA export factors were mutated.[41,42] For unspliced HIV-mRNA, this phenomenon is especially well documented.[45,46,48–50] In these studies, it was shown that conditions where cytoplasmic transport of HIV-mRNAs is prevented do not lead to nuclear RNA accumulation, but rather to a decrease in the stability of the transcripts. As transcription, splicing and nuclear export are coupled processes, inhibition of nuclear export probably leads to reduced transcription and/or cotranscriptional degradation of the affected cellular RNAs. A direct effect of LMB on transcription seems unlikely, as the drug has no effect on overall RNA levels in Drosophila cells.[40] Likewise, we did not observe a general reduction in total or cytoplasmic RNA concentration in various LMB-treated cell lines (data not shown).

Cytoplasmic accumulation of some of the investigated sequences was also reduced by expression of an inhibitory fragment of Nup214. The phenotypes of inhibition by this fragment and by LMB are not identical, however, as some RNAs behave differently under the two conditions. This is likely to result from the different mechanisms of down-regulation of the CRM1-pathway: LMB is expected to covalently modify and thereby inhibit virtually all cellular CRM1. The Nup214-fragment, in contrast, competes with endogenous Nup214 for CRM1-binding. The absolute levels of inhibition by LMB and the Nup214-fragment are therefore very different and are likely to affect different mRNAs to different extents. This may also explain the reduced levels of many mRNAs in the nuclear fraction of LMB-treated cells, which was not observed in the transfection experiments. Also, LMB may have other effects on CRM1 that are not directly linked to its function in nuclear export.[51,52]

Does the CRM1-dependence of the identified messages reflect their CRM1-dependent nuclear export, or does CRM1-inhibition simply lead to reduced transcription during T cell activation? Since activation of T cells is mediated by the transcription factors NFAT and NF-κB, which are established targets of the CRM1 export pathway,[22,53] LMB could have indirect effects by changing the subcellular localization of these factors. A negative effect of LMB on NFAT or NF-κB-dependent transcription,

however, could not be observed.[54,55] Similarly, LMB had no effect on the transcription of COX-2, one of the putative CRM1-export targets.[29] In a detailed analysis of CD83, one of our isolated sequences, in a heterologous system (i.e. independent of transcription factors that are involved in T cell activation), we found a 40–50% reduction in the expression level when CRM1 was inhibited. This result shows that the expression of CD83 is sensitive to inhibition of the CRM1 export pathway, in its natural context as well as in a heterologous system. A higher level of inhibition could not be obtained, probably because cells with complete inhibition of CRM1 do not survive and were therefore excluded from the analysis. Of course, we cannot exclude the possibility that the CD83 mRNA can be exported by alternative mechanisms when expressed from a cDNA plasmid at high levels.

In a parallel study (unpublished results†), a posttranscriptional response element (PRE) was identified in the coding sequence of CD83 that mediates CRM1-dependent expression. This *cis*-acting element binds to HuR, a member of the ELAV family of AU-rich element (ARE) RNA-binding proteins.[56,57] HuR has been suggested to function in CRM1-dependent c-fos-mRNA export, together with the NES-containing proteins pp32 and APRIL.[28] According to a recent microarray study of HuR-bound mRNAs,[58] six additional mRNAs from our study interact with HuR, either *via* an ARE (TNFα, BIRC3 and LTA) or *via* a novel degenerate U-rich motif (CD82, NINJ1 and BCL2L1). These mRNAs could be exported by CRM1 in a HuR-dependent fashion, like CD83. In this context, it is interesting to note that the HuR-interacting adenoviral protein E4orf6 seems to redirect several ARE-containing mRNAs from a CRM1-dependent to a CRM1-independent export pathway.[59] Adapter proteins distinct from the HuR/pp32/APRIL-system could be involved in CRM1-dependent export of other mRNAs.

Taken together, T cells appear to switch to the CRM1-pathway for export of some mRNAs transcribed from induced genes, possibly to expedite their expression. It will be interesting to investigate whether TAP-dependent mRNA export is concomitantly repressed. In T cells, HIV takes advantage of CRM1-dependent transport for nuclear export of some of its mRNAs. CRM1-dependent mRNA export appears to be involved in other systems of cellular activation or differentiation, e.g. the maturation of dendritic cells expressing CD83. RDA, together with microarray analysis, should allow the identification of such mRNAs that take an unusual nuclear export pathway.

† Prechtel, A. T., Chemnitz, J., Schirmer, S., Ehlers, C., Langbein-Detsch, I., Stulke, J. *et al.* (2006). Expression of CD83 is regulated by HuR via a novel *cis*-active coding region RNA element. *J. Biol. Chem.* Feb 16; Epub ahead of print. PMID: 16484227.

Materials and Methods

Cell culture

HeLa cells, 293T cells, COS-7 cells and Cl-4 cells (CV-1 cells from African green monkey that are stably transfected with an HIV-1 BH10 env expression plasmid[35]) were grown on plastic dishes in Dulbecco's modified Eagle's medium. Jurkat Tag cells (Jurkat cells expressing the large T antigen of simian virus 40[60]) were grown in suspension in RPMI 1640 medium. Media contained 10% (v/v) fetal calf serum (FCS), 100 units/ml of penicillin, 100 μg/ml of streptomycin and 2 mM glutamine. All tissue culture reagents were from Gibco-BRL. Jurkat cells were first serum-starved overnight and then stimulated with 10% FCS for 3 h. Subsequently, they were treated with or without 20 nM LMB for 30 min, followed by the addition of ionomycin (1 μM final concentration, added from a 1 mM stock solution in DMSO) and phorbol-12-myristate 13-acetate (PMA; 50 ng/ml, added from a 0.5 mg/ml stock solution in ethanol) and further incubation for 3 h.

RNA isolation

A total of 2×10^7–2×10^8 cells were lysed in NP-40 buffer (10 mM Hepes–KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 20% (v/v) glycerol, 1 mM DTT, 0.5% NP-40 for HeLa and Cl-4 cells, 0.25% NP-40 for Jurkat Tag cells) on ice for 2 min. After centrifugation at 470*g* for 5 min at 4 °C, lysed cells were resuspended in NP-40 buffer, centrifuged as above and the supernatants were combined. The NaCl concentration was adjusted to 140 mM, 2.8 volumes of ethanol and 4 volumes of RLT buffer (Qiagen, RNeasy Midi Kit) were added, and cytoplasmic RNA was isolated according to the instructions of the manufacturer. The nuclear pellet was washed twice with wash buffer (10 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 140 mM NaCl, 20% glycerol, 1 mM DTT) and resuspended in RLT buffer. After addition of one volume of 70% (v/v) ethanol, samples were loaded onto RNeasy midi columns and further purifications were performed according to the Qiagen protocol. Poly(A)$^+$ RNA was isolated from total RNA with Oligotex mRNA kit (Qiagen) and quantified fluorimetrically with SYBRgreen dye (Molecular Probes) as described.[61]

Northern blotting

A 200 ng sample of Cl-4 poly(A)$^+$ RNA or 5 μg of total RNA from Jurkat cells was separated by formaldehyde agarose gel electrophoresis and transferred onto a Biodyne B membrane (Pall, UK). Hybridizations were carried out overnight at 42 °C in 6× SSC, 5× Denhardt, 0.5% (w/v) SDS, 100 μg/ml of salmon sperm DNA and 50% formamide. A fragment of HIV-1 pNL4-3 env (accession no. AF324493; nucleotides 8092–8965) was used as radioactive probe. Probes for human GAPDH and β-actin were obtained by RT-PCR from HeLa RNA using specific primers (GAPDH: 5′-GTGAAGGTCG GAGTCAACGG and 5′-CCTGGTGACCAGGCGC; β-actin: 5′-GAGCGGTTCCG CTGCCCTGAGGCACTC and 5′-GGGCAGTGATCTC CTTCTGCATCCTG). Other probes were derived from isolated DpnII-fragments. Probes were labeled with the Prime It II Kit (Stratagene) according to the manufacturer. Blots were analyzed by autoradiography.

cDNA synthesis and representational difference analysis

First and second strand cDNA synthesis and cDNA RDA using 0.5–1 μg of cDNA were performed as described.[31] cDNA was quantified with Picogreen dsDNA Quantification Reagent (Molecular Probes). The following primers were used for cDNA-RDA: R-Bam-24: 5′-AGCACTCTCCAGCCTCTCACCGAG; R-Bam-12: 5′-GATCCTCGGTGA; J-Bgl-24: 5′-AC CGACGTCGACTATCCATGAACA; J-Bgl-12: 5′-GA TCTGTTCATG; N-Bgl-24: 5′-AGGCAACTGTGCTATCC GAGGGAA; N-Bgl-12: 5′-GATCTTCCCTCG. For nuclear RDAs (i.e. nuclear RNA was used as starting material), *tester* representations were obtained from HeLa cells or Cl-4 cells that had been incubated in the presence of 20 nM LMB for 4 or 13 h. Here, representations derived from untreated cells served as *driver*. For cytoplasmic RDAs (i.e. cytoplasmic RNA was used as starting material), *tester* representations were obtained from cells that had been incubated in the absence of LMB. Here, the *driver* was generated from cells that had been incubated with LMB for 24 h. For generation of difference products 1, 2 and 3, *tester* to *driver* ratios of 1:100, 1:800 and 1:10,000 were used, respectively.

RDA with cytoplasmic RNA from activated Jurkat T cells that had been treated with or without LMB for a total of 3.5 h was performed with the following modifications:[62] for generation of difference products 1, 2 and 3, *tester* to *driver* ratios of 1:10, 1:100 and 1:5000 were used. Mung bean nuclease treatment was omitted and the first PCR reaction after substractive hybridization was used in a 1:20 dilution in the second PCR reaction. Instead of using J-Bgl-24/12 primers for generation of the third difference product, the 24-mer (5′-AGACAGTGCCGGATGTAGCCATAA) and 12-mer (5′-GATCTTATGGCT) were used.

Final RDA difference products were cloned into pGem-T Easy (Promega), sequenced with T7 or SP6 primers and identified by Blast searches.

cDNA Southern blotting

Aliquots of 1.5–3 μg of *driver* or *tester* representation were separated by agarose gel electrophoresis and blotted onto Biodyne B membranes. Hybridizations were carried out overnight at 65 °C in Church buffer (0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA, 1% (w/v) BSA). Probes for env, β-actin and GAPDH were used as above. RDA fragments were used for the detection of the indicated clones. African green monkey rRNA from Cl-4 cells was detected using probes corresponding to nucleotides 2567–2840 of the human 28 S sequence (accession no. M11167) and to nucleotides 1195–1746 of the human 18 S sequence (accession no. K03432), respectively. Blots were analyzed by autoradiography and *tester* to *driver* ratios were quantified with the Quantity one software (Biorad).

RT-PCR

RT-PCR was performed with equal amounts of nuclear or cytoplasmic RNA. After treatment of RNA with RNase-free DNase (RQ1; Promega), reverse transcription was carried out with the SuperScript II RNase H^- Reverse Transcriptase Kit (Invitrogen). PCR reactions were carried out with the following primers: IL2RA (30 cycles), 5′-TCGGAACACAACGAAACAAG and 5′-GTGACGAGG CAGGAAGTCTC; LTA (30 cycles), 5′-CCCACCAGTGG CATCTACTT and 5′-GACCCCTGAAATGGTCAGAA; BIRC3 (30 cycles), 5′-CCGGAAGAATAGAATGGCACT-3′ and 5′-TCCGCAATTGTTCTTCCACT-3′; TNFα (31 cycles), 5′-AGCCCATGTTGTAGCAAACC and 5′-GGAAGACCCCTCCCAGATAG; GAPDH (24 cycles), 5′-GTGAAGGTCGGAGTCAACGG and 5′-CCTGG TGACCAGGCGC.

For quantitative RT-PCR detecting HIV-env-sequences, 400 ng of poly(A)$^+$ RNA from nuclear or cytoplasmic fractions of cells that had been treated with or without LMB was reverse transcribed in the presence of [α-^{32}P]dCTP, using oligo-dT as a primer. The quality of the cDNA was assessed by agarose gel electrophoresis of 500 cpm of cDNA, followed by autoradiography. Equal amounts of cDNA were subjected to LightCycler-PCR (Roche), using the HIV-env oligonucleotides 5′-GAGTAGCACCCACCAAGGCA-3′ and 5′-CCCAA-GAACCCAAGGAACAA-3′ and the SYBR Green I kit (Roche) for detection. LightCycler software 3.5 was used for signal quantification. For quantification of CD83 and Gαs-sequences, equal amounts of total cytoplasmic (2.2 μg) or total nuclear RNA (0.4 μg) from LMB-treated or untreated cells were reverse transcribed and 1% of the resulting cDNA was subjected to LightCycler analysis. Signals were normalized for a corresponding β-actin-signal. Primers were 5′-AGATCGAGAAGCAGCT GCA-3′ and 5′-CTTTGGTTGCCTTCTCACC-3′ for Gαs, 5′-GCATGGAACGAGCTTTTCTC-3′ and 5′-GCTGCAT ACATCGCTGAAAA-3′ for CD83 and 5′-GAGCGGT TCCGCTGCCCTGAGGCACTC-3′ and 5′-GGGCAGTG ATCTCCTTCTGCATCCTG-3′ for β-actin. For all quantitative PCR reactions, standard curves were derived by serial dilutions of corresponding plasmids (HIV-env) or PCR products (CD83, Gαs, β-actin).

Fluorescence *in situ* hybridization

A 28 S-rRNA-RDA clone in pGem-T Easy was linearized with NcoI and used as template for *in vitro* transcription with SP6 polymerase (MAXIscript; Ambion) in the presence of digoxigenin-11-UTP (Roche). The protocol for fluorescence *in situ* hybridization (FISH) was adapted from Dirks *et al*.[63] Cells grown on coverslips were fixed for 20 min in 3.7% formaldehyde, 10% acetic acid and 155 mM NaCl, treated with 10 μg/ml of proteinase K for 5 min and fixed again with 1% formaldehyde for 5 min. Digoxigenin-labeled *in vitro* transcripts were hybridized at a concentration of ~10 μg/ml in 50% formamide, 5× SSC, 50 μg/ml of yeast tRNA, 50 μg/ml of heparin (Sigma) at 60 °C overnight. After hybridization, cells were washed twice for 5 min at 60 °C in 50% formamide, 5× SSC, 0.5% SDS, 50% formamide, 2× SSC and once in TBST (10 mM Tris–HCl (pH 8), 150 mM NaCl, 0.05% Tween 20). The probe was detected with a fluorescein-labelled sheep anti-digoxigenin Fab fragment (Roche) at a dilution of 1:200. Cells were mounted in HistoGel (Linaris) and analyzed by fluorescence microscopy using an Olympus IX70 inverted fluorescence microscope. Pictures were processed using Adobe Photoshop 6.0. Nucleolar and cytoplasmic fluorescence was quantified using NIH Image.

Transfection of Jurkat cells, cell sorting and PCR analysis

For inhibition of the CRM1-dependent export pathway, 1.5×10^7 Jurkat Tag cells were transfected with 30 μg of pcDNA3-CANc[64] or pcDNA3, respectively, together with

15 μg of pcDNA3-eGFP vector using Lipofectamine 2000 (Invitrogen). After 20 h, the cells were serum-starved for 24 h and then stimulated with serum for 3 h and then with ionomycin and PMA as above for an additional 2 h. A total of 2×10^6 eGFP positive cells were sorted using the Becton Dickinson FACS-ARIA™ Cell Sorter. Either total RNA (1/3 of cells) or RNA from the cytoplasmic or nuclear fraction as obtained upon lysis of the cells in NP-40 buffer (2/3 of cells; see above) was isolated using Trizol reagent (Gibco BRL). RNA samples were analyzed by RT-PCR using the first strand cDNA (AMV) synthesis kit (Roche Molecular Biochemicals) and the following oligonucleotides for amplification:

S14: 5′-GGCAGACCGAGATGAATCCTC and 5′ CA GGTCCAGGGGTCTTGGTCC; CD83, 5′-GGTGAAGGT GGCTTGCTCCGAAG and 5′-GAGCCAGCAGCAGGA CAATCTCC; NINJ1, 5′-TCTATG TGCCCCTGGTGGTC and 5′-CAGTCAGAGCAAGGGCTGGT; IL-2, 5-′ TA CAAAGAAAACACAGCTACAACTGGA and 5′-TGAT ATTGCTGATTAAGTCCCTGG; c-fos, 5′-AGATCCCT GATGACCTGGGC and 5′-CGTGTAAGCAGTGCAG CTGG; GM-CSF, 5′-CAGCCACTACAAGCAGCACT and 5′-CTTCTGCCATGCCTGTATCA; GNG4, 5′-GGGGCAG TAGAATGAAAGAGG and 5′-GGATGGGTGTTGGTCT CACT; BCL2L1, 5′-GTAAACTGGGGTCGCATTGT and 5′-TGTCTGGTCATTTCCGACTG; BIRC3, 5′-CCGGAA GAATAGAATGGCACT and 5′-TCCGCAATTGTTCTTC CACT. Primers for TNFα, LTA and IL2RA were as above. PCR-reactions were performed with 25 (S14), 26 (CD83, c-fos, NINJ1, GNG4), 27 (IL2R, BIRC3, LTA), or 34 cycles (TNFα, BCL2, GM-CSF, IL-2).

Metabolic labelling

De novo CD83 synthesis was analyzed in COS-7 cells, which were mock-transfected or cotransfected with 500 ng of either pcDNA3-CANc or the empty vector pcDNA3, in combination with 250 ng of p3UTR-CD83 expression vector. At ∼60 h posttransfection, cells were washed with cysteine/methionine-free medium containing 10% dialyzed FCS and incubated in the same medium for 1 h. After labelling for 30 min using 200 μCi of [^{35}S]Translabel (1175 Ci/mmol; MP Biomedicals), cells were pelleted, washed twice in PBS, and lysed in EIA buffer (0.1% NP-40, 150 mM NaCl, 50 mM Hepes (pH 7.3)). For deglycosylation, the lysates were incubated with PNGase F (New England Biolabs) according to the manufacturer's instructions. Equal amounts of cellular lysates were subjected to CD83-specific immunoprecipitation analyses using a monoclonal anti-CD83 antibody (clone HB15a; Acris) and analyzed by 12% (w/v) SDS-PAGE followed by autoradiography.

Transfection of 293T cells and FACS analysis

293T cells were transfected in duplicate in 12-well plates with 100 ng each of pcDNA3-eGFP and pcDNA3-CD83, together with either 1 μg of the empty pcDNA3 vector or 1 μg of pcDNA3-CANc. After three days, cells were harvested, suspended in PBS containing 1% BSA and stained with monoclonal anti-CD83 (HB15e, Serotec) as primary and Alexa 647 goat-anti-mouse (Molecular Probes) as secondary antibody. After extensive washings and fixation with 1% formaldehyde in PBS, cells were analyzed for GFP-fluorescence (FL1) and CD83-fluorescence (FL4) by flow cytometry, using the Becton Dickinson FACS-Calibur. Expression levels for CD83 were normalized to GFP-fluorescence and expressed as a percentage of the control.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.02.041

The supplementary data comprises one Figure showing the time-course of mRNA expression.

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