Ratjadone and leptomycin B block CRM1-dependent nuclear export by identical mechanisms

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Abstract Research on the export of proteins and nucleic acids from the nucleus to the cytoplasm has greatly gained from the discovery that the actinobacterial toxin leptomycin B (LMB) specifically inactivates the export receptor chromosomal region maintenance 1 (CRM1). Recently, it was shown that myxobacterial cytotoxins, named ratjadones (RATs), also bind to CRM1 and inhibit nuclear export. However, the reaction mechanism of RATs was not resolved. Here, we show that LMB and RAT A employ the same molecular mechanism to inactivate CRM1. Alkylation of residue Cys528 of CRM1 determines both LMB and RAT sensitivity and prevents nuclear export of CRM1 cargo proteins.

Keywords: Ratjadone; Leptomycin B; Chromosomal region maintenance 1; Nuclear export

1. Introduction

Communication between the cell nucleus and the cytoplasm frequently requires shuttling of signal transducers such as Smads or Stats between both compartments [1,2]. It is well established now that a group of related proteins, called karyopherins, mediates many of the necessary translocation processes (reviewed in [3]). It is a hallmark of karyopherin-assisted translocation that the cargo molecules harbour loosely conserved localisation signals in their primary sequence. Generally, these signals are necessary and sufficient for the karyopherins to recognise their cargo. For nuclear import, stretches of positively charged residues termed nuclear localisation signal are required, whereas nuclear export signals (NESs) consist of hydrophobic residues, predominantly leucines (reviewed in [4]). The karoypherins are also termed importins or exportins according to the direction of transport that they support. At present, there are no pharmacological inhibitors available to inactivate importins. Karyopherin-mediated nuclear export, on the other hand, is readily blocked by the actinobacterial biocide leptomycin B (LMB), which was isolated from *Streptomyces* sp. [5]. The discovery of the export blocking activity of LMB has tremendously advanced the study of protein nucleocytoplasmic shuttling and lead to the discovery of the exportin chromosomal region maintenance 1 (CRM1). CRM1 was originally identified as a protein responsible for maintaining the chromosome structure in the yeast *Schizosaccharomyces pombe* [6]. Later, it was found that CRM1 acted also as the NES receptor in both lower and higher eukaryotes (reviewed in [7]). Recognition of the NES by CRM1 is specifically inhibited by LMB, which binds covalently to a single sulfhydryl group of CRM1 [8,9]. The modification of Cys529 of Crm1 from *S. pombe* and of the homologous Cys528 from *H. sapiens* was demonstrated to block association of CRM1 with the NES-containing cargo, thus inhibiting nuclear protein export. It has been recognised over the last years that NES-mediated nuclear export by CRM1 is a universal and conserved mechanism by which the subcellular localisation of proteins is determined in cells (reviewed in [7]). Very recently, a group of polyketides from myxobacterium *Sorangium cellulosum* was also implicated in the CRM1-dependent export of proteins from the nucleus. Hauser and colleagues convincingly demonstrated that the ratjadones (RATs), which are structurally related to LMB, inhibit the formation of nuclear export complexes by the direct binding of RAT to CRM1 ([10]; for a review on RATs, see [11]). However, the mechanism by which the RATs inactivate CRM1 was not resolved. Here, we demonstrate that RAT A blocks protein export by covalently binding to Cys528 of human CRM1. Thus, both LMB and RATs inhibit nuclear protein export by identical reaction mechanisms.

2. Materials and methods

2.1. Cell culture

HeLaS3 cells were grown on glass coverslips at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS; Biochrom, Berlin) and antibiotics. For microinjections, the coverslips were transferred into 3.5 cm dishes filled with 1 ml culture medium without or with the indicated export inhibitor. LMB (Sigma) and RAT A (a kind gift of Dr. Markus Kalesse, Universität Hannover) were dissolved in methanol

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Abbreviations: CRM1, chromosomal region maintenance 1; GFP, green fluorescent protein; GST, glutathione *S*-transferase; LMB, leptomycin B; NES, nuclear export signal; RAT, ratjadone; Smad, a combination of the gene names from *C. elegans*, Sma, and *D. melanogaster*, Mad; Stat, signal transducer and activator of transcription

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(10 ng/μl) and both used in culture medium at a final concentration of 10 ng/ml. Transient transfections were performed by using Lipofectamine-plus (Life Technologies) according to the manufacturer's instructions.

2.2. Plasmid construction

pGST-green fluorescent protein (GFP) and pGST-NES-GFP containing a canonical NES from human Stat1 (residues 365–427) were described [12]. A mammalian expression vector encoding a fusion protein of GFP with a Stat1-derived NES activity (aa 365–427) was constructed by PCR-amplification of the cDNA of NES-GFP from pGST-NES-GFP using the following primers: 5′-ATATATGGATCCAGATAAAGATGTGAATGAG-3′ and 5′-CGCCCCGACACCCGCCAACACCC-3′ (restriction sites underlined). After restriction digestion with the enzymes *Bam*HI and *Eco*RI, the resulting fragment encoding the Stat1 NES was inserted into the *Bgl*II/*Eco*RI sites of the vector pEGFP-C3 (Clontech) to yield pGFP-NES. The cDNAs for wild-type CRM1 and the CRM1 mutant Cys528 to Ser (TGT>TCT) were kindly provided by Dr. Bryan R. Cullen (Duke University, Durham) and Dr. Shigeki Miyamoto (University of Wisconsin), respectively. To allow for indirect immunodetection of CRM1, FLAG-tagged expression constructs were prepared as follows. The wild-type and mutant CRM1 coding sequences were amplified by PCR using the primer pair 5′-ATTATAGGATCCATGCCAGCAATTATGACAATG-3′ and 5′-ATATTAGGATCCCATCACACATTTCTTCTGG-3′. The resulting cDNA was digested with *Bam*HI and inserted into the *Bgl*II sites of the vector pFLAG, which was derived from pStat1-Flag [12] after removing the Stat1 coding region by digestion with *Eco*RI. pFLAG allows the expression of a C-terminal FLAG epitope (DYKDDDDK) followed by a stop codon. PCR was carried out with Vent-proofreading enzyme (NEB). The constructs were confirmed by DNA sequencing across the mutated codons and the restriction sites used for cloning.

2.3. Microinjections and fluorescence analysis

Preparation of the glutathione *S*-transferase (GST)–GFP fusion proteins and microinjections were performed as described in Begitt et al. [13]. In brief, the fusion proteins were expressed in *Escherichia coli* BL21 and affinity-purified using glutathione-sepharose beads (Amersham). The recombinant proteins (1 μg/μl in microinjection buffer: 20 mM HEPES–OH, pH 7.5, 110 mM KAc, 0.5 mM EDTA, 5 mM DTT) were injected into the nucleus of HelaS3 cells using a Transjector 5246 (Eppendorf). They were co-injected with TRITC-coupled BSA (0.2 μg/μl; Sigma) to indicate the site of injection. Approximately fifty cells were injected within 15 min. After injection, the cells were incubated at 37 °C for another hour before fixation for 15 min in 4% (v/v) formaldehyde in PBS. Nuclei were stained with Hoechst 33258 (5 μg/ml in PBS; Sigma) for 3 min. After washing three times with PBS and once with H_2O, the cells were mounted in Dako fluorescent mounting medium (DakoCytomation). Cells were examined by conventional fluorescence microscopy using an Axioplan 2 Imaging system (Zeiss).

2.4. Immunocytochemistry

For immunodetection of FLAG-tagged CRM1, a mouse monoclonal antibody directed against the FLAG epitope (M2; Sigma) was employed. Twenty-four hours after transfection, the cells were fixed for 15 min in 4% (v/v) formaldehyde/PBS, then permeabilised with 0.2% (v/v) Triton X-100 and blocked for 30 min with 10% FCS (v/v) in PBS. Incubation with the primary antibody at a dilution of 1:1000 was done in 10% FCS in PBS at 4 °C overnight. After repeated washing with PBS, the cells were incubated for 1 h at RT with a Cy3-conjugated donkey anti-mouse IgG antibody (Jackson Immunoresearch) at a dilution of 1:2000 in 10% FCS in PBS. Following Hoechst staining and extensive washing with PBS, the cells were mounted as described above.

2.5. Mass spectrometric analysis

Peptide labelling was essentially performed as described by Kudo et al. [9]. One μg of a human CRM1 peptide (residues 513–530; Biosynthan, Berlin) containing the conserved Cys528 was treated with or without 10 μg RAT in 15 μl labelling buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl). Following overnight incubation at 37 °C, the samples were analysed by mass spectrometry. Samples were diluted with 5 μl of 1% (v/v) formic acid in water and purified over a C18 reversed-phase microcolumn, ZipTip (Millipore), according to the manufacturer's manual. Peptides were eluted from the ZipTips with 5 μl of 70% (v/v) acetonitrile, 0.25% (v/v) formic acid in water. Mass spectrometric analyses of unmodified and modified CRM1 peptides were performed as previously described [14,15]. Briefly, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc.) using an alpha-cyano-4-hydroxycinnamic acid matrix. In order to verify the sequences of CRM1(513–530) and the position of modification, MS/MS analyses were performed by nanoelectrospray quadrupole TOF (Q-Tof Ultima, Manchester, UK) and MALDI TOF-TOF (4700 Proteomics Discovery System; Applied Biosystems) mass spectrometry.

3. Results and discussion

At first, we demonstrated that RAT A can revert the accumulation in the cytoplasm of a protein that contains a canonical NES. A reporter protein consisting of GFP fused to a canonical NES, amino acids 365–427 of human Stat1 [12], was expressed in mammalian cells. The steady state localisation of this protein is cytoplasmic, contrary to GFP with a pancellular or predominantly nuclear localisation (compare Fig. 1A and D). Due to their small size (30 kD), these proteins are capable of passing through the nuclear pore by free diffusion [16]. Treatment of cells expressing GFP for 60 min with 10 ng/ml LMB or 10 ng/ml RAT A was without detectable influence on the subcellular distribution of GFP (Fig. 1E and F). On contrary, the same treatment abolished the cytoplasmic accumulation of GFP-NES, which was now found in both the nucleus and the cytoplasm (Fig. 1B and C).

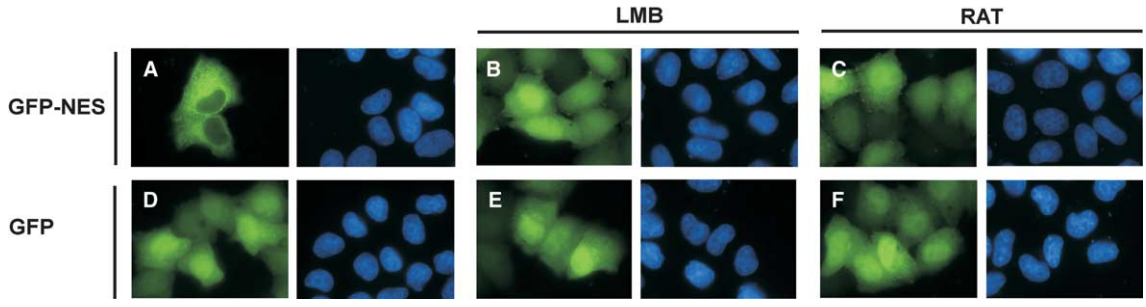

Fig. 1. RAT A causes nuclear accumulation of a NES-containing reporter protein. HelaS3 cells were transiently transfected with pGFP-NES (A–C) or with a GFP expression vector (D–F). Twenty-four hours after transfection, cells were treated with LMB (B, E) or RAT A (C, F) for 1 h or left untreated (A, D). Subsequently, cells were fixed with formaldehyde and stained with Hoechst 33258 to indicate the nuclei (blue). The GFP signal is depicted in green.

A microinjection assay was used to specifically reveal the inhibitory effect of LMB and RAT A on nuclear export. Here, a recombinant reporter protein consisting of a fusion of GST and GFP was purified from bacteria and subsequently injected into the nucleus of HeLa cells. Its large size (55 kD) precluded free diffusion across the nuclear envelope (Fig. 2A). The insertion of cDNA coding for a Stat1 NES activity (aa 365–427) between the genes coding for GST and GFP resulted in a bacterial expression construct that yielded the export reporter protein GST–NES-GFP. As is shown in Fig. 2B, a 1 h incubation period following nuclear microinjection of the export reporter resulted in its steady state cytoplasmic accumulation. Preincubation of the cells with RAT A or LMB for 3 h before microinjection completely blocked the subsequent nuclear export of the NES fusion protein (Fig. 2C and D). These results confirm the previous observation that both bacterial metabolites inhibit the nuclear export of NES-containing proteins at a similar concentration [10].

A major unanswered question concerns the reaction mechanisms of RATs and LMB, both of which target CRM1. The molecular mechanism of CRM1 inactivation was identified as the addition of LMB through its α,β unsaturated δ-lactone to the sulfhydryl group of residue Cys528. To investigate whether Cys528 was targeted also by RATs, which similar to LMB contain an unsaturated lactone, we followed a procedure that was employed by Kudo et al. [9] to demonstrate the modification by LMB of Cys528 of human CRM1. An 18mer peptide (residues 513–530) corresponding to the stretch of conserved hydrophobic amino acids in the N-terminal flanking region of Cys528 (residues 517–528) was synthesised and reacted without or with RAT A. Mass spectrometric analysis of the unreacted material revealed a mass peak of the unmodified CRM1 peptide at *m/z* 2103.06. After the incubation with RAT A, an additional peak at *m/z* 2559.40 was detected which

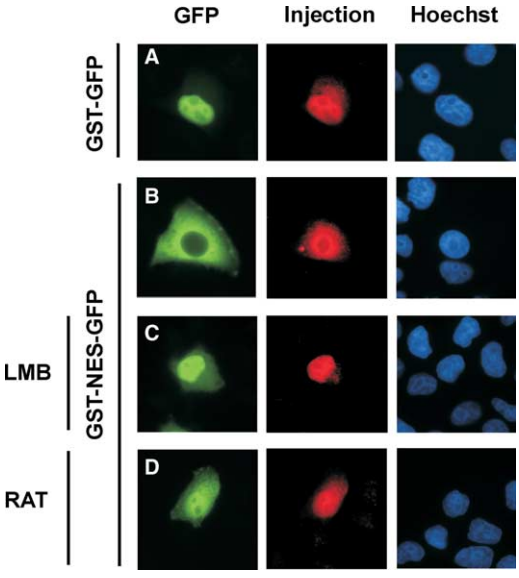

Fig. 2. RAT A blocks the nuclear export of a NES-containing reporter protein. The indicated GST–GFP fusion proteins were injected into the nuclei of HelaS3 cells. One hour after microinjection, cells were fixed and stained with Hoechst 33258 to show the nuclei. The site of injection is given by the coinjected injection marker (TRITC-BSA). The GFP signal is shown in green. In C and D, cells were preincubated with the indicated export inhibitor for 3 h before microinjection.

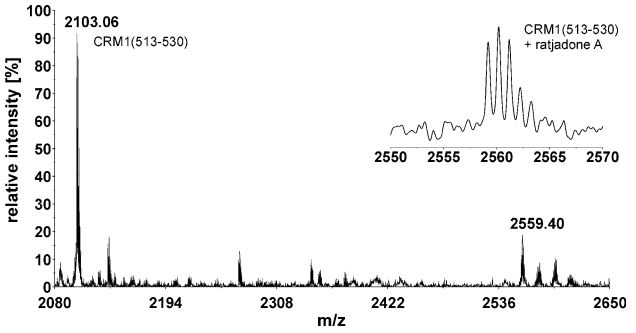

Fig. 3. RAT A covalently binds to Cys528 of a CRM1-peptide. Synthetic human CRM1 (residues 513–530) that contains Cys528 was incubated with RAT A for 24 h at 37 °C and analysed by MALDI–TOF-MS. The peaks with *m/z* 2103.06 (theoretical *m/z* 2103.19) and 2559.40 (theoretical *m/z* 2559.48; see also enlargement) correspond, respectively, to the unmodified and RAT A-alkylated hCRM1 sequence ^{513}EKRFLVTVIKDLLGLCEQ^{530}.

corresponds to the adduct of the peptide and RAT A (Fig. 3). The amino acid sequence and the site of RAT modification was confirmed by tandem MS. The evidence for the alkylation of Cys528 comes from the presence of unmodified N-terminal b ions and the neutral loss of the RAT moiety during fragmentation (data not shown).

We next analysed a mutant of human CRM1 with a serine mutation in position 528. Cysteine528 is conserved between *S. pombe* and humans, which are LMB-sensitive, but not in LMB-insensitive organisms such as *Sac. cerevisiae*. Moreover, replacement of this cysteine by serine resulted in insensitivity of *S. pombe* CRM1 towards LMB [8,9]. For our experiments, Flag-tagged expression constructs of wild-type or mutant human CRM1 were co-expressed with GFP-NES in HeLa cells. As was described before for NIH3T3 cells [17], CRM1 was localised mainly intranuclear or at the nuclear rim. Strongly overexpressing cells displayed also cytoplasmic staining. In this respect, no differences were observed between mutant and wild-type CRM1 (Fig. 4 and data not shown). Moreover, the localisation of CRM1 was not sensitive towards LMB or RAT (Fig. 4). On contrary, treatment of cells with either LMB or RAT A precluded the cytoplasmic accumulation of GFP-NES in cells expressing wild-type CRM1 (Fig. 4A) and a pancellular distribution of the reporter protein resulted (Fig. 4C and E). Expectedly, expression of the Ser528 mutant of CRM1 rendered cells insensitive to the activity of LMB, since the cytoplasmic accumulation of the NES-containing reporter persisted (Fig. 4B and D). Importantly, the identical phenotype was observed also after treatment with RAT A in the cells expressing the mutant CRM1 (Fig. 4F). Thus, we conclude that both LMB and RAT inhibit protein transportation in vivo by targeting residue Cys528 of CRM1. Given their identical and highly specific modes of molecular action, we propose that RAT A and LMB can be used interchangeably in the study of protein export from the nucleus.

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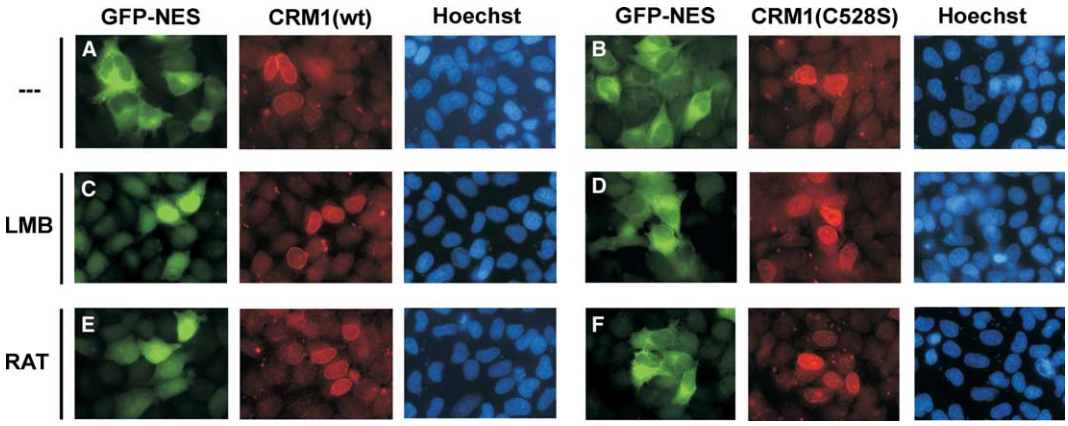

Fig. 4. Mutation of cysteine 528 to serine renders CRM1 resistant to both LMB and RAT A. HelaS3 cells were cotransfected with GFP-NES and either wild-type CRM1 (A, C, E) or the mutant CRM1(C528S) (B, D, F). Twenty-four hours later, the cells were treated with RAT A or LMB (10 ng/ml each) for 3 h before fixation with formaldehyde. FLAG-tagged CRM1 was detected using a mouse monoclonal anti-FLAG antibody. The distribution of GFP-NES and the positions of Hoechst-stained nuclei are shown.

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