Table 3. Expression patterns of genes reported to be differentially expressed in intact versus damaged regions of cartilage as well as in normal versus OA cartilage*

Comparison, expression pattern	Ref.	Gene name (alias)	Gene symbol	Fold change†	SEM	*P*
Intact versus damaged regions of cartilage from the same joint						
Intact < damaged	4	Nerve growth factor, β polypeptide (NGF)‡	*NGFB*	5.08	1.54	0.011
	4	Neurotrophic tyrosine kinase receptor type I (p140 TrkA)	*NTRK1*	ND		
	5	Insulin-like growth factor binding protein 3	*IGFBP3*	5.94	2.71	0.056
	5	Insulin-like growth factor binding protein 4‡	*IGFBP4*	3.93	1.20	0.006
	5	Insulin-like growth factor binding protein 5	*IGFBP5*	1.17	0.33	0.611
	6	Heparan sulfate proteoglycan 2 (perlecan)	*HSPG2*	2.22	0.80	0.074
Intact > damaged	7	Matrix metalloproteinase 13	*MMP13*	−9.51	9.34	0.32
	7	Hyaluronan and proteoglycan link protein 1 (link protein)	*HAPLN1*	−1.94	0.45	0.043
	7	B cell CLL/lymphoma 2	*BCL2*	1.05	0.34	0.828
	7	Sex determining region Y–type high mobility group box 9	*SOX9*	1.08	0.18	0.685
Normal versus OA cartilage						
Normal < OA	34	Tumor necrosis factor α–induced protein 6 (TSG-6)‡	*TNFAIP6*	25.34	5.78	0.001
	35	Serine protease 11 (HtrA1)‡	*PRSS11*	3.08	0.77	0.012
	36	Prostaglandin E synthase‡	*PTGES*	12.44	3.08	0.013
	37	S100 calcium binding protein A4‡	*S100A4*	5.33	1.73	0.007
	37	Fibronectin 1‡	*FN1*	6.16	1.49	0.002
	37	Transforming growth factor β–induced 68 kd (BIGH3)‡	*TGFBI*	12.05	3.74	0.001
	37	Collagen, type I, α2‡	*COL1A2*	5.67	3.19	0.027
	37	Matrix metalloproteinase 2‡	*MMP2*	−7.30	2.90	0.017
	14	Chordin-like 2 (CHL2)‡	*CHRDL2*	−8.95	5.35	0.06
Normal > OA	38	Serine proteinase inhibitor, clade E, member 1 (PAI-1)‡	*SERPINE1*	14.38	8.09	0.016

* OA = osteoarthritic; ND = not detected.
† Positive numbers represent high expression in damaged regions; negative numbers represent high expression in intact regions.
‡ Included in the list of 114 genes selected by our criteria. Complete data for all of the selected genes are available upon request from the corresponding author.

be smaller than that estimated using oligonucleotide array analysis.

Expression patterns of genes reported to be differentially expressed in intact versus damaged regions of OA cartilage and in normal versus OA cartilage. We assessed the microarray data for genes that in previous studies were shown to be differentially expressed in intact versus damaged regions of OA cartilage from the same joint or in normal versus OA cartilage from different patients (4–7,14,34–38). The name and symbol of the reported genes are shown in Table 3. Table 3 also shows the sample characterization (comparison being made) and the gene expression pattern reported in previous studies, as well as the results of oligonucleotide array analysis for each gene.

Consistent with studies that compared intact and damaged regions of OA cartilage from the same joint, we confirmed high expression of *NGFB* (4), *IGFBP3*, *IGFBP4* (5), and *HSPG2* (6) in the damaged region, as well as high expression of *MMP13* and *HAPLN1* (7) in the intact region of OA cartilage. However, we did not observe *NTRK1* gene expression itself or differential expression of *IGFBP5*, *BCL2*, and *SOX9*.

We also evaluated whether the results of comparisons between intact and damaged regions of cartilage from the same joint resembled the results of comparisons between normal and OA cartilage obtained from different individuals. At least 7 genes reported to be highly expressed in OA cartilage compared with normal cartilage (*TNFIP6* [34], *PRSS11* [35], *PTGES* [36], *S100A4*, *FN1*, *TGFBI*, and *COL1A2* [37]) were highly expressed in the damaged region compared with the intact region of OA cartilage. However, there were some exceptions. According to our data, the 2 genes reported to be highly expressed in OA cartilage (*MMP2* [37] and *CHRDL2* [14]) were highly expressed in the intact region, and *SERPINE1* (38), which was reported to be expressed at low levels in OA

cartilage, was highly expressed in the damaged region of OA cartilage.

DISCUSSION

In this study, we compared the gene expression profiles in intact versus damaged regions of OA cartilage. Transcripts with a ≥2-fold difference in mRNA expression between these 2 regions accounted for an average of 8% of all expressed transcripts per OA cartilage tissue sample, ~10% of which were commonly detected in the 5 patient samples. The former observation indicates that the gene expression profile of chondrocytes in the intact region is quite different from that of chondrocytes in the damaged region, even though both regions are in contact with the same synovial fluid and have the same genetic background. The latter finding suggests that the gene expression profiles of chondrocytes in OA cartilage change in a region-specific manner. This also suggests the presence of common molecular mechanisms of OA development, assuming that the changes in gene expression patterns of chondrocytes lead to cartilage degeneration.

Using our selection criteria, we identified 114 genes that were differentially expressed in the intact region versus damaged region of OA cartilage. Thirty-five of these genes were up-regulated in the intact region, and 79 genes were up-regulated in the damaged region. The validity of our data was confirmed using real-time quantitative PCR and comparing our findings with those of previous studies. The possible roles of these genes with significantly altered mRNA expression are discussed here in terms of the reaction patterns of chondrocytes in order to determine the underlying mechanisms that may participate in the pathogenesis or progression of OA. Sandell and Aigner (39) described 5 categories of cellular reaction patterns related to OA development: phenotypic modulation of articular chondrocytes, formation of osteophytes, chondrocyte proliferation and apoptosis, matrix synthetic activity of chondrocytes, and matrix degradation activity of chondrocytes. The latter 3 are discussed below.

With regard to chondrocyte proliferation and apoptosis, the *CCND1*, *PSAT*, and *S100A4* genes are known to be involved in cell proliferation, as mentioned above (17,18,21). These genes were more highly expressed in damaged regions than in intact regions of OA cartilage. This finding is consistent with the pathologic feature showing some clusters of chondrocytes in the surface layer of damaged cartilage, and it supports the hypothesis that changes in gene expression patterns of chondrocytes lead to the pathologic condition of OA in cartilage. Yet, among the 114 genes we identified, no genes were clearly related to apoptosis. (Complete data for all of the selected genes are available upon request from the corresponding author.) However, we cannot suggest that there are no differences in the activation of the apoptosis signaling pathway between intact and damaged regions of OA cartilage, because the proteolytic cascade, rather than transcriptional regulation, is important in apoptotic signaling (40).

With regard to the matrix synthetic activity of chondrocytes, our microarray data showed that although the signal intensities of type II collagen and aggrecan were very high in both intact and damaged regions of OA cartilage, there were no differences in gene expression between these 2 regions (data not shown). However, we found that 3 interstitial collagen genes (*COL1A1*, *COL1A2*, and *COL5A1*) and 4 genes for enzymes involved in the collagen biosynthetic pathway (*P4HA3*, *LOXL2*, *LEPREL1*, and *LOXL3*) were highly expressed in the damaged region of OA cartilage. Interestingly, hierarchical clustering analysis showed that 4 of these 7 genes (*COL1A2*, *COL5A1*, *LOXL2*, and *LOXL3*) belonged to the same cluster as proliferation-associated genes (*S100A4* and *PSAT1*). This suggests that wound healing, including the process of cell proliferation and interstitial collagen synthesis, occurs in damaged OA cartilage, where the expression of genes related to wound healing might be regulated in the same manner.

With regard to the matrix degradation activity of chondrocytes, the most obvious pathologic feature in the damaged region is advanced cartilage destruction. Therefore, we predicted that expression levels of protease genes would be higher in damaged regions than in intact regions. Consistent with this expectation, we identified 4 proteases (*DKFZP586H2123*, *ADAMTS6*, *ADAM12*, and *PRSS11*) with high expression in the damaged region of OA cartilage among the selected 114 genes. However, MMP-2, which can degrade the extracellular matrix, is only one protease showing high expression in the intact region of OA cartilage. In particular, the *PRSS11* (HtrA1) gene was recently reported to enhance cartilage degeneration via digestion of major cartilage components (41), and the single-nucleotide polymorphisms of the *ADAM12* gene are associated with the progression of knee OA (28). However, there is a discrepancy between the function of the detected genes and the histopathologic features. The expression levels of 3 genes known to inhibit degradation of the extracellular matrix (*TNFAIP6*, *SERPINE1*, and *TIMP3*) were

significantly high in the damaged region compared with the intact region of OA cartilage. These molecules are probably up-regulated to prevent the progression of cartilage destruction in the damaged areas of OA cartilage.

We subsequently examined whether the results of comparisons between intact and damaged regions of OA cartilage from the same joint resembled the results of comparisons between normal and OA cartilage obtained from different persons. We found that comparisons between normal and OA cartilage and between intact and damaged regions of OA cartilage yielded similar results with regard to the expression pattern of 7 of the 10 genes examined (Table 3). This suggests that during the transition from normal cartilage to OA lesional cartilage, the gene expression profile changes before there is any apparent damage to the cartilage. Because the expression levels of some genes probably change during the transitional period from normal to normal-appearing cartilage, our study design might have allowed us to miss some important genes that show no differences in expression levels between intact and damaged regions. To overcome this problem, further studies comparing these samples with normal cartilage samples from normal joints are needed.

What causes the OA-specific pattern of gene expression? Several possible mechanisms have been investigated, such as mechanical stress, cytokine stimulation, cell–matrix interaction, hypoxia, and reoxygenation. One of the strongest candidates is mechanical stress, because the damaged cartilage region is usually subjected to mechanical loading, whereas the intact region is not. In particular, chondrocytes residing in damaged regions are susceptible to mechanical stress because the tensile properties of the damaged cartilage are lost as a result of destruction of the collagen network (42). Proinflammatory cytokines, especially interleukin-1 (IL-1) and TNFα, are also closely related to the development of OA (3). We thought that these cytokines might be accessible to the chondrocytes in damaged cartilage. However, some IL-1–induced genes were detected in both regions (in the intact region, *ETS1*, *GUCY1A3*, *C4BPA*, *PBEF*, and *APOD*; in the damaged region, *TNFAIP6*, *PTGES*, *FN1*, *NGFB*, and *TNFSF11*). Furthermore, although MMP-2 mRNA is not significantly up-regulated by treatment with IL-1 (43), our microarray data showed that MMP-2 mRNA was expressed 7.3-fold higher in intact regions than in damaged regions of OA cartilage. Therefore, the effects of the cytokine alone could not account for these complex conditions of OA in vivo. Up-regulation of MMP-2 mRNA might be the result of other factors, including hypoxia/reoxygenation, cell–matrix interactions, and intermittent hydrostatic pressures (44–46).

In conclusion, our study demonstrated a clear difference in the gene expression profile in damaged regions of human OA cartilage compared with that in intact regions. The pattern of differences between these 2 regions was similar among 5 pairs of OA cartilage samples. This finding implies that there is a common mechanism responsible for the destruction and maintenance of the articular cartilage in OA. Elucidation of this mechanism is important for the development of effective treatments for OA.

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Role of synoviolin in rheumatoid arthritis: possible clinical relevance

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The symptoms of rheumatoid arthritis (RA) are derived from the process of chronic inflammation and the overgrowth of synovial cells. However, the mechanisms of RA flares are not fully understood. To clarify these mechanisms, the authors carried out immunoscreening using anti-rheumatoid synovial cell antibodies and identified and cloned synoviolin – an endoplasmic reticulum-associated degradation (ERAD) E3 ubiquitin ligase. This molecule is overexpressed in the rheumatoid synovium and approximately 30% of littermates of *synoviolin*-overexpressing mice developed spontaneous arthropathy. Moreover, *synoviolin*$^{+/-}$ mice were resistant to collagen-induced arthritis through enhanced apoptosis of synovial cells. Based on the gain- and loss-of-function, the authors consider synoviolin to play a critical role in the crisis of arthritis, and propose that RA is a hyper-ERAD disease. These findings provide a new pathogenetic model of RA, and suggest that synoviolin could be targeted as a therapeutic strategy for RA.

Keywords: apoptosis, endoplasmic reticulum-associated degradation, endoplasmic reticulum stress, hyper-endoplasmic reticulum-associated degredation disease, rheumatoid arthritis, synovial cell, synoviolin

future Medicine

Rheumatoid arthritis (RA) is a disease associated not only with painful joints, but also generalized symptoms related to the whole body such as febricula, malaise and anorexia. RA affects approximately 1% of the population worldwide [1,2]. Although RA is a serious condition, a specific cure that leads to the improvement of quality of life is not yet available, mainly because the exact etiology of RA is still poorly understood.

The pathological features of RA include the chronic inflammation of systemic joints associated with overgrowth of synovial cells, which eventually causes cartilage and bone destruction in the affected joint [3,4]. It is thought that inflammation results from the activation of the cytokine system regulated by inflammatory cells [5]. During the course of inflammation, activated macrophages produce tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6. These cytokines, in turn, stimulate overgrowth of synovial cells to form a mass of synovial tissue called pannus, which invades the bone and cartilage through osteoclast activation and protease production [6–9]. As RA is considered to be an autoimmune disease, medical treatment targeting inflammation has been applied. However, nearly 25% of RA patients do not respond to anticytokine or anti-inflammatory therapies [10–12]. This article will focus on synovial cells and discuss the mechanisms of hyperplasia of rheumatoid synovial cells (RSCs).

Molecular cloning of synoviolin

The authors' laboratory first cloned RSCs and found that these cells bear autonomous proliferation properties with aberrant cytokine production in a culture system [13,14]. Moreover, it was found that human T-cell leukemia virus type I (HTLV-I), one of the epidemic human retroviruses associated with arthropathy (HAAP) [15], and *tax*, the viral transforming gene that causes HAAP and its product, pp40Tax, could transform synovial cells into RSCs in patients and overexpressing mice [16–18]. However, expression of pp40Tax is not observed in human RSCs. Thus, to determine the functionally equivalent endogenous gene products in RSCs, comprehensive immunoscreening was carried out using anti-RSC antibodies, and synoviolin was cloned successfully [19], a human homolog of the yeast ubiquitin ligase (E3) Hrd1p/Del3p [20]. Synoviolin is an endoplasmic reticulum (ER)-resident membrane protein with a RING-H2 motif and is highly expressed in the rheumatoid synovium [19]. Since it is expected that this endogenous molecule might elucidate the cause of RA, the distribution and functional properties of synoviolin was investigated further.

Arthropathy in *synoviolin*-overexpressing mice

To investigate physiologically the role of this molecule, the tissue distribution of synoviolin was first examined by northern blot analysis. The results in mice showed that *synoviolin* was ubiquitously expressed in various tissues. This was an unexpected result, since synoviolin was cloned in RSCs and it was predicted that *synoviolin* was specifically expressed in the rheumatoid synovium.

To gain insight into the function of synoviolin *in vivo*, the *synoviolin*-overexpressing mouse was generated. Essentially, it is desirable to analyze the

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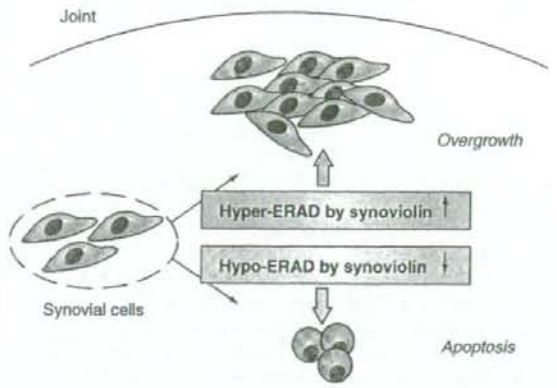
Figure 1. RA is a 'hyper-ERAD disease'.



Synoviolin is highly expressed in RSCs, which leads to hyperactivation of the ERAD system. Synoviolin is an E3 ubiquitin ligase and functions in the ERAD system. When the misfolded proteins accumulate in the ER they are eliminated by ubiquitin- and proteasome-dependent degradation processes to avoid cell death caused by dysfunction of ER. The ERAD system is activated by overexpression of synoviolin, which prevents apoptotic death of synovial cells, consequently leading to synovial hyperplasia.
ER: Endoplasmic reticulum; ERAD: ER associated degredation; RA: Rheumatoid arthritis; RSC: Rheumatoid synovial cell.

synoviolin function by using a promoter expressed specifically in synovial cells. However, such a promoter has not yet been identified. Thus, human *synoviolin*-overexpressing mice were established using a β-actin promoter, which drives systemic protein expression, including synovial cells. Strikingly, approximately 30% of *synoviolin*-overexpressing mice developed spontaneous arthropathy with marked joint swelling even in the C57BL/6 strain. Interestingly, no other abnormality was apparent in these mice throughout their life, apart from the spontaneous arthritis. In spite of the ubiquitous expression of *synoviolin*, this phenotype of *synoviolin*-overexpressing mice exhibited pathological features similar to those of patients with RA, indicating that these mice are a suitable animal model of RA.

Collagen-induced arthritis model of *synoviolin*-deficient mice

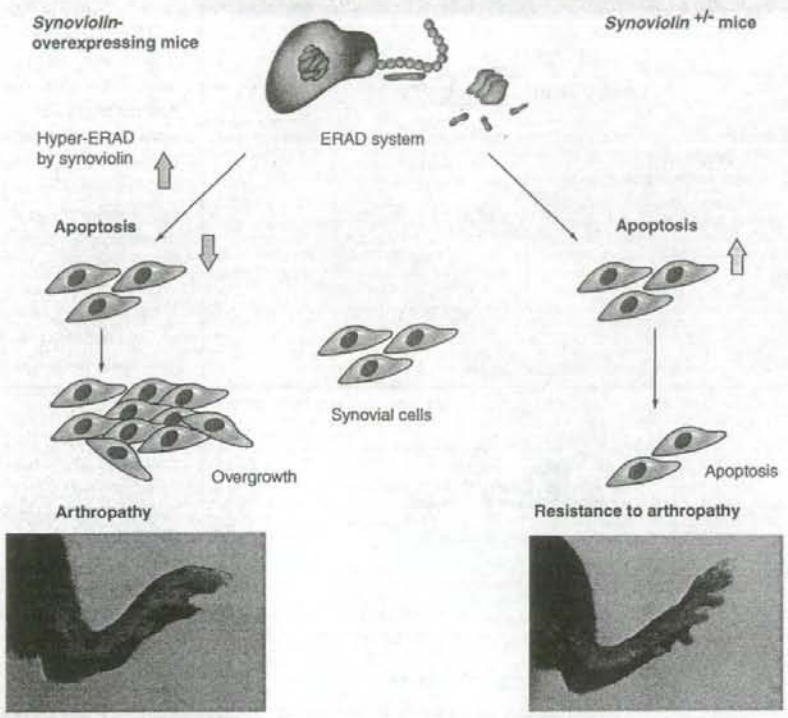
The results of the gain-of-function study suggested that synoviolin acts as an inducer of synovial cell hyperplasia. An attempt to verify this hypothesis was subsequently made in *synoviolin*-deficient mice, that is, loss-of-function. Mice with type II collagen-induced arthritis (CIA) are used commonly as a model of experimental arthritis. This model can also be considered suitable for analyzing the function of synoviolin in RA. Thus, *synoviolin*-deficient ($syno^{-/-}$) mice were generated by gene-targeted disruption.

Unfortunately, all fetuses lacking synoviolin died *in utero* at around E13.5 [21], although Hrd1p/Del3p, a yeast ortholog of synoviolin, was described as nonessential for survival [22]. $Syno^{-/-}$ were anemic owing to enhancement of apoptosis in the fetal liver [21]. In the next series of studies, the relationship between synoviolin and flares of RA was clarified. The CIA model was applied to *synoviolin*-heterozygous mice ($syno^{+/-}$). The results showed that $syno^{+/-}$, treated for induction of CIA, were resistant to the development of arthritis. Furthermore, CIA-$syno^{+/-}$ mice exhibited intact immunoreactions and demonstrated inflammatory cell infiltration but lacked advanced synovial cell hyperplasia [19]. These results indicate that the synovial cell hyperplasia process is independent of proceeding immunoreactions, and is an indispensable process in the pathogenesis of arthropathy. This conclusion was confirmed *in vitro*; *synoviolin* small interfering RNA (siRNA) suppressed the growth of synovial cells even when these cells were stimulated by cytokines [19]. When combined, these results of loss-of-function indicate that synoviolin is essential for the crisis of arthritis.

New disease concept: hyper-ERAD disease

How does synoviolin, an E3 ubiquitin ligase resident in the endoplasmic reticulum (ER), participate in synovial cell hyperplasia? In the ER of eukaryotic cells, newly synthesized proteins are transported for correct folding. Under normal conditions, the transport and folding processes in the ER match the requirement of the secretory pathway. Alternatively, since various environmental insults can overwhelm the efficacy of intracellular protein folding, cells have a self-protective mechanism for survival following an increased demand for protein folding. ER stress can trigger a cellular response termed the unfolded protein response (UPR) [23], during which the synthesis of new proteins is inhibited globally and genes encoding the ER chaperone proteins are also upregulated to refold the misfolded proteins correctly [24]. However, when the UPR fails to deal with this problem, misfolded proteins are eliminated by ubiquitin- and proteasome-dependent degradation processes, known as the ER-associated degradation (ERAD) system, and thus spare

Figure 2. Drug targets for treatment of RA.



Gain-of-function of synoviolin results in the development of spontaneous arthropathy through the anti-apoptotic effects of synoviolin. On the other hand, *syno*$^{+/-}$ mice are resistant to the CIA model. Therefore, synoviolin could be a novel therapeutic target for RA.
CIA: Collagen-induced arthritis; ERAD: Endoplasmic reticulum associated degredation
RA: Rheumatoid arthritis.

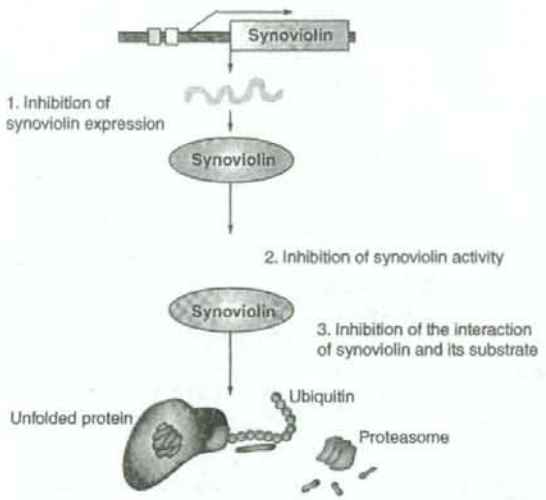
the cells from cell death through ER dysregulation [25–27]. Synoviolin is involved in this ERAD system as an ubiquitin ligase [19,28,29] and takes part in rescuing the cells from cell death. In synovial cells of CIA-*syno*$^{+/-}$, which have impaired the ERAD system owing to the lack of synoviolin, the apoptotic cells are significantly increased [19]. Therefore, in CIA-*syno*$^{+/-}$, synovial cells cannot avoid apoptotic cell death because of lack of synoviolin, thus preventing synovial cell overgrowth. However, in RSCs, hyperactivation of the ERAD system by overexpression of synoviolin could prevent synovial cell apoptotic death, consequently leading to synovial hyperplasia.

Dysfunction of the ERAD system has been implicated in various disorders. For instance, production of expanded polyglutamine causes certain inherited neurodegenerative disorders [30–32]. Furthermore, mutation of the *parkin* gene, a well known ubiquitin ligase protein in the ERAD system, is thought to result in neuronal death of the substantia nigra in patients with autosomal recessive juvenile parkinsonism [33]. The authors first postulated that hyperactivation of the ERAD system could lead to proliferative diseases and then introduced a novel concept, the hyper-ERAD disease (Figure 1).

Possible drug target for the treatment of RA

It was demonstrated that the etiology of RA could be based on hyperactivation of the ERAD system due to overexpression of synoviolin and it was concluded that synoviolin could be considered as

Figure 3. Development of synoviolin-based therapy for rhematoid arthritis.



Possible pathways in which synoviolin could be targeted for the treatment of rheumatoid arthritis.

a novel therapeutic target of RA (Figure 2). In making synoviolin a therapeutic target, three methods can be considered (Figure 3).

First, overexpression of synoviolin in RSCs could lead to the suppression of hyper-ERAD. In order to control the amount of synoviolin, it is important to elucidate the transcriptional regulation of synoviolin. Clarification of the mechanisms of transcriptional regulation of synoviolin should allow the suppression of transcription of synoviolin, thus avoiding a hyper-ERAD state. The authors recently identified a crucial site for *synoviolin* expression in the *synoviolin* proximal promoter, in other words, the Ets binding site, and that the growth-associated binding protein (GABP)-α/β complex is essential for its transcriptional regulation [34]. Thus, it is expected that RA gene therapy will become possible in the future.

Second, synoviolin is an E3 ubiquitin ligase that acts in the ERAD system, which suggests inhibition of its enzymatic activity. Blockade of synoviolin enzymatic activity should be associated with suppression of the hyper-ERAD state. In this regard, human *synoviolin*-overexpressing mice with a lack of enzymatic activity did not show any phenotype, including arthritis [Unpublished Data]. Therefore, there is a need to develop synoviolin inhibitors.

Third, since synoviolin is an E3 ubiquitin ligase, one could intercept synoviolin-substrate interaction. Synoviolin cannot function as an enzyme in the absence of an interaction with the substrate. Moreover, it is conceivable that not only synoviolin but also its substrate are expressed highly or specifically in RSCs. This is supported by the results of studies in *synoviolin*-overexpressing mice only showing arthritis despite the systemic expression. However, a substrate of synoviolin has not yet been identified. Thus, there is a need for further studies to identify a synoviolin substrate. In this regard, the authors are currently conducting such studies using the yeast two-hybrid system [Yamasaki S *et al.*, Zhang L *et al.*, Unpublished Data]. If a specific substrate of synoviolin in RSCs is identified, it is expected that disturbances of the synoviolin–substrate interaction could be used to prevent RA flares. Moreover, it is predicted that this may enhance the development of antibody therapies.

In any case, since CIA was almost completely suppressed in *syno*$^{+/-}$, further studies should be conducted to investigate the impact of approximately 50% inhibition of the amount and/or activity of synoviolin. Moreover, since synovial cell outgrowth is a common event in RA, new drugs designed to block *synoviolin* expression/activity might help find the cure of RA.

The development of a synoviolin-based marker for the diagnosis of RA, together with the development of drugs designed to block synoviolin expression and/or activity could perhaps allow the identification of the disease at an early stage and administration of effective therapy for RA.

Conclusion

Synoviolin is an important causative factor of RA, and our findings could open new avenues of investigation into the pathogenesis of RA.

Future perspective

RA has a negative impact on quality of life. Cytokines released from immune cells cause chronic inflammation and stimulate the proliferation of synovial cells that destroy bone and cartilage of joints. However, nearly 25% of RA patients do not respond to anticytokine or anti-inflammatory therapies. This may be because synovial cells acquire the autonomous proliferation ability that could be controlled by an ERAD-associated E3 ubiquitin ligase synoviolin. Therefore, we believe

that our findings will help design novel therapies for RA and expect development of selective inhibitors of synoviolin in the future.

Moreover, bone and cartilage destruction of joints in RA is one of the most serious terminal symptoms, thus we are considering the possibility of synoviolin involvement in these phenomena. Clarification of this point is our next subject, and we hope our research will help in the establishment of a new therapy.

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Executive summary

Background

- Rheumatoid arthritis (RA) affects approximately 1% of the adult population worldwide.
- RA is a disease associated with generalized symptoms related to the whole body especially to painful joints.
- The burden of musculoskeletal diseases on society has been recognized throughout the world and RA is defined as one of the most important diseases in the Bone and Joint Decade, launched by the World Health Organization in 2000 to reduce the social and financial costs of musculoskeletal disorders to society.

Pathology

- RA includes chronic inflammation of systemic joints associated with overgrowth of synovial cells, which eventually causes cartilage and bone destruction in the joints.

Synoviolin

- Cloning of synoviolin from rheumatoid synovial cells ocurred using immunoscreening.
- Synoviolin is an endoplasmic reticulum (ER)-resident membrane protein.
- Synoviolin is an E3 ubiquitin ligase associated with ER-associated degredation (ERAD).
- Synoviolin is expressed ubiquitously, however it is highly expressed in the rheumatoid synovium.

Animal study

- Mice overexpressing *synoviolin* exhibit spontaneous arthropathy and a progressive synovial hyperplasia characteristic of RA patients.
- Reduced expression of *synoviolin* in mice correlated with protection from arthritis. This resistance is not due to an impaired cytokine response or reduced inflammatory cell infiltration, but to an increase in synovial cell apoptosis.

New disease concept

- RA is an ERAD activated disease caused by by overexpressed *synoviolin* in rheumatoid synovial cells.

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The nuclear import of RNA helicase A is mediated by importin-α3

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Abstract

RNA helicase A (RHA), an ATPase/helicase, regulates the gene expression at various steps including transcriptional activation and RNA processing. RHA is known to shuttle between the nucleus and cytoplasm. We identified the nuclear localization signal (NLS) of RHA and analyzed the nuclear import mechanisms. The NLS of RHA (RHA-NLS) consisting of 19 amino acid residues is highly conserved through species and does not have the consensus classical NLS. In vitro nuclear import assays revealed that the nuclear import of RHA was Ran-dependent and mediated with the classical importin-α/β-dependent pathway. The binding assay indicated that the basic residues in RHA-NLS were used for interaction with importin-α. Furthermore, the nuclear import of RHA-NLS was supported by importin-α1 and preferentially importin-α3. Our results indicate that the nuclear import of RHA is mediated by the importin-α3/importin-β-dependent pathway and suggest that the specificity for importin may regulate the functions of cargo proteins.

Keywords: RNA helicase A; Nuclear localization signal; Importin

RNA helicase A/nuclear DNA helicase II (RHA) was isolated as a human homologue of *Drosophila* maleless (MLE) involved in sex-specific gene dosage compensation of fruit fly [1–3]. It belongs to the DExH family of ATPase/helicase and unwinds both double-stranded DNA (dsDNA) and RNA (dsRNA) [4,5]. RHA contains several functional domains. The amino terminus has two double-stranded RNA-binding domains (dsRBD1 and dsRBD2). The catalytic core domain, containing seven well-conserved motifs and ATP binding site, is located within the central region. The minimal transactivation domain (MTAD) is next to the ATP binding site and the carboxyl terminus contains glycine-rich single-stranded nucleic acid-binding domain (RGG) [6,7].

RHA displays various functions at several stages of gene expression. For example, it is involved in transactivation in an ATP-dependent manner and/or functions as a bridging factor [8]. We showed previously that RHA mediates the recruitment of RNA polymerase II (Pol II) through MTAD to cAMP-responsive element binding protein (CREB)-binding protein (CBP) and enhancement of cAMP-mediated transcriptional activation [6]. RHA also mediates various transactivation, such as BRCA1 [9], the activation function 1 domain of mineralocorticoid receptor (MR) in a ligand-selective manner [10], and nuclear factor κB (NF-κB) [11]. In addition, recent studies showed that RHA directly binds to promoters such as the *cis*-acting transactivation response element (TAR) of HIV-1 [12],

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the *p16INK4a* promoter [13], and multidrug resistance gene (MDR1) via MEF1 [14]. These studies indicated that RHA has important roles in the transcription of several genes.

RHA localizes predominantly in the nucleus. It is also known that RHA is a shuttling protein and regulates the gene expression in the cytoplasm. In the nucleus, RHA plays roles in post-transcriptional regulation. It is included in spliceosome and involved in the processing of transcripts [15]. Furthermore, it promotes the viral gene expression and the export of the constitutive cytoplasmic transport element (CTE) containing viral mRNA. Thus, the translocation of RHA between the nucleus and cytoplasm seems to be regulated in a transcription-dependent manner [16,17]. In the cytoplasm, RHA is reported to relate with translation. These studies indicate that RHA plays a common role in the expression of a wide variety of genes at various steps.

The transport of proteins between the cytoplasm and the nucleus occurs through nuclear pore complexes (NPCs) in the nuclear envelope. These proteins contain specific sequences, the nuclear localization signal (NLS), required for the nuclear import. SV40 large T-antigen (T-NLS) and nucleoplasmin have the classical NLSs and are well analyzed. They contain one or more clusters of basic amino acids, particularly lysine, which are important for their activity. Other types of NLSs have also been identified such as M9 sequence of heterogeneous nuclear ribonucleoprotein (hnRNP) A1, which bears no sequence similarity to classical NLSs [18,19].

The nuclear import of classical NLSs is mediated by a heterodimeric receptor complex composed of importin-α and -β. Importin-α is responsible for binding to the NLS, while importin-β mediates binding of the transport complex to the NPC. Importin-α interacts with importin-β through its N-terminal importin-β-binding (IBB) domain, is rich in basic amino acids, and functions as an adapter molecule [20,21]. In addition to the import receptors, a small GTPase Ran is required for nuclear import pathway. In the cytoplasm, the GTPase-activating protein (RanGAP) hydrolyzes GTP bound with Ran to GDP and Ran-GDP rapidly is imported into the nucleus. The nuclear Ran is created by asymmetric distribution of regulatory factors for Ran. The nuclear exchange factor RCC1 (Ran GEF) promotes the exchange of Ran-GDP to Ran-GTP. Ran-GTP binds to the import receptor at the nuclear side of NPC and causes the dissociation of the NLS-import receptor complex [22–24].

Only one gene coding for importin-β has been identified. In contrast, six isoforms of importin-α have been identified in human cells, whereas there is one importin-α gene in *Saccharomyces cerevisiae*. The importin-α isoforms are classified into three groups based on their sequence homology. The first group contains importin-α1/Rch1. Although importin-α2 has similarity with importin-α1, it is found in *Xenopus laevis* and other vertebrates but not in mammals. The second group has importin-α3/Qip1 and α4/hSRP1γ, and they have 85% sequence identity. The third group consists of importin-α5/NPI-1, α6, and α7, which has 80% homology. Although there are differences in their expression levels, almost all importins are expressed ubiquitously. Previous studies showed that members of the importin-α family have different substrate specificities [25–27].

Previous study reported that the transport of RHA is mediated by the domain of 110 amino acids at its C-terminus (termed NTD for nuclear transport domain), and that the import and export activities of this domain can be separated. It is also suggested that the nuclear import of NTD is importin-α/β-dependent [16]. Moreover, it is reported that methylation of NTD by protein arginine methyltransferase 1 (RPMT1) regulates the import of RHA [28]. However, the involvement of importin-α and -β has neither been demonstrated in binding studies nor in vitro nuclear import assays. For further understanding of the nuclear import of RHA, we identified in the present study the NLS of RHA and characterized its nuclear import mechanisms.

Materials and methods

Transfection. For in vivo expression experiments, HeLa cells were seeded onto 14-mm^2 square coverslips and incubated for 24 h before transfection. Transfections were performed by using FuGENE 6 reagent (Roche Diagnostics) with 0.5 μg of each construct, according to the protocol provided by the manufacturer. Cells were incubated for 24 h after transfection, washed three times with PBS, and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. After removal of formaldehyde, cells were washed with PBS and water, then the coverslips were mounted and analyzed by TCS4D confocal laser microscopy system (Leica).

Construction of plasmids. The plasmids encoding the enhanced green fluorescent protein fusion protein (pEGFP-RHA) were constructed by inserting the RHA fragments into pEGFP-C2 (Clontech Laboratories). The fragments of RHA1, 2, 3, and 4 regions were obtained from pGEX RHA1, 2, 3, and 4 [8], respectively, and inserted into pEGFP-C2. To create the expression plasmid encoding the EGFP-pyruvate kinase (PK) fusion protein, the fragment corresponding to the PK sequence was generated by PCR. The pyruvate kinase fragment was inserted into pEGFP-C2 and named pEGFP-PK. To generate the deletion mutants, the fragments were generated by PCR and termed RHA 4b, RHA C4, RHA N2, RHA NC2, and RHA NC4, respectively. These fragments were subcloned into pEGFP-PK. For mutational analysis, substitutions of arginine or lysine with alanine were introduced into the RHA4b region by sequential PCR steps. The plasmids encoding GFP-RHA R1160A, K1163A, and R1166A were constructed by inserting the fragments of RHA derived from pEGFP-RHA and the corresponding mutated RHA4b fragments into pEGFP-C2.

For glutathione *S*-transferase pull-down assay, the fragments of deletion mutants and point mutated RHA were inserted into the bacterial expression vector pGEX-5X-1 (Amersham Biosciences). The control plasmids GST-M9-GFP and GST-T-NLS-GFP were described previously [29]. To generate pGEX-5X-1-GFP for import assay, the coding sequence of EGFP was amplified by PCR and inserted into pGEX-5X-1. For GST-RHA-NLS-GFP, a PCR fragment corresponding to RHA-NLS was inserted into pGEX-5X-1-GFP and pGEX-5X-1. To express the biotinated importin proteins as probes, importin-α1 and importin-β fragments were obtained from pGEX-2T-PTAC58 [30] and pGEX-2T-PTAC 97 [31], respectively. Fragments of other importin families were generated by RT-PCR. These fragments were subcloned into PinPoint-Xa-3 (Promega).

Expression and purification of recombinant proteins. GST fusion proteins were expressed in *Escherichia coli* (*E. coli*) strain BL21 (DE3) and purified with glutathione–Sepharose beads (Amersham Biosciences), using the instructions provided by the manufacturer. To prepare import substrates for the in vitro import assays, purified GST proteins were eluted from beads and concentrated. The eluted proteins were purified with Sephadex 75 (Amersham Biosciences). For in vitro import assay,

expression and purification of recombinant importin-αs and importin-β proteins were performed as described previously [32]. The recombinant wild type and G19V Ran were expressed, purified, and charged with GDP and GTP, respectively, as described previously [29]. As probes for the binding assay, importin-αs and -β were expressed and labeled with biotin in *E. coli*. The cells were suspended into lysis buffer (20 mM Hepes, pH 7.3, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 μg/ml of aprotinin, leupeptin, and pepstatin A). The lysates were centrifuged to remove cell debris and used for probes.

In vitro import assay. Nuclear import assays were performed essentially as described previously [33,34]. Total cytosol from Ehrlich ascites tumor cells was prepared as described previously. For the competition experiments, biotinylated BSA, which was chemically coupled to a synthetic peptide containing the T-NLS (T-BSA) [34] and recombinant IBB [35], was prepared as described previously and added to the reaction mixtures as an unlabeled competitor.

Pull-down assay. The bacterial lysates containing biotin-tagged importin-α and -β were diluted fivefold with Probe dilution buffer (20 mM Hepes, pH 7.3, 1 mM EDTA, 1 mM DTT, 0.0625% Tween 20, 6.25% glycerol, and protease inhibitors). The diluted bacterial lysates were precleaned by incubation with GST bound to glutathione–Sepharose beads in binding buffer (20 mM Hepes, pH 7.3, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween 20, 5% glycerol, and protease inhibitors) for 1 h at 4 °C. After centrifugation, supernatants were incubated with 2 μg of GST fusion proteins immobilized on glutathione–Sepharose beads for 4 h at 4 °C. At the end of incubations the beads were washed four times with binding buffer and bound fractions were separated by SDS–PAGE. Recovered importin-αs and -β were detected by using streptavidin conjugated with horseradish peroxidase (HRP) (Amersham Biosciences).

Result

NLS of RHA consists of 19 amino acids

It is reported that RHA contains the bidirectional nuclear transport domain in its C terminal region. The region includes 110 amino acids mapped to 1150–1259 [16]. To understand further the nuclear import mechanism of RHA, we identified the minimal sequence necessary for the NLS activity. A series of deletion mutants were constructed and expressed as GFP fusion proteins in HeLa cells (Fig. 1A). The C terminal region of RHA which comprises 1138–1270 amino acids and contains NTD (RHA 4b) was localized in the nucleus, while the mutant which has the region between 1155 and 1172 deleted (RHAΔ4b) was localized in the cytoplasm (Fig. 1B). As indicated in previous studies, this region has nuclear import activity.

To identify the minimal region required for nuclear import of RHA, we generated another series of deletion mutants of RHA 4b. The mutants were termed RHA C2 (aa 1138–1173), N2 (1173–1270), NC2 (1155–1222), and NC4 (1155–1173) (Fig. 1C). To prevent the nuclear entry by passive diffusion, mutants were expressed as a fusion protein with GFP-pyruvate kinase. PK has been used as a reporter protein for NLS identification because it is normally located in the cytoplasm and can localize to the nucleus when attached to a functional NLS. As shown as Fig. 1D, GFP-PK was localized in the cytoplasm. Three mutants containing the NC4 region accumulated in sufficient amounts in the nucleus, while RHA N2 was localized in the cytoplasm. To test the importance of the NC4 region in the context of full-length RHA, NC4 region was deleted and monitored for the effect on GFP-RHA localization. This mutant deleted the NC4 region from RHA full length (RHA ΔNLS) as well as RHA Δ4b showed cytoplasmic localization (Fig. 1B). These results indicate that the region extending from amino acid 1155 to 1173 is required for

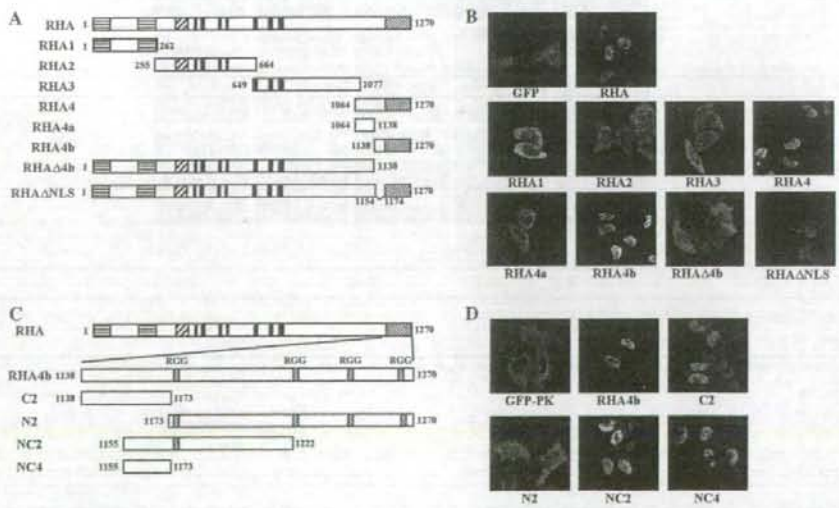

Fig. 1. NLS of RHA (RHA-NLS) consists of 19 amino acid residues. (A,C) Schematic representation of RHA and deletion mutants fused to the C-terminus of GFP. Relative position of individual domains of RHA. Solid boxes indicate the functional domain, the N-terminal double-stranded RNA binding domain (dsRBD), transactivation domain, central core helicase domain (I–VII), and C-terminal RGG box (B,D). Fluorescent microscopy of wild type (wt) and deletion mutants of RHA expressed in HeLa cells as GFP-fusion proteins.

human	1150	YGDGPRPPKMARYDNGSGY
bovin	1147	YGDGPRPPKMARYDNGSGY
mouse	1151	YGDGPRPPKMARYDNGSGY
fruit fly	1165	FSDGGGPPKRGRFETGRFT
SV40		PKKKRKV
nucleoplasmin		KRPAAIKKAGQAKKKKLD
c-Myc		PAAKRVKLD
RanBP3		PPVKRERTS

Fig. 2. RHA-NLS is highly conserved through species. The amino acid sequences of the RHA-NLS region in RHA homologues.

nuclear localization of RHA. The NC4 region consists of 19 amino acids and does not include RGG motifs. It has no significant similarity to other known NLSs such as the classical monopartite NLS, bipartite NLS or M9 of hnRNP A1 (Fig. 2).

Nuclear import of RHA is mediated by importin-α/β complex

It is reported that the nuclear import of RHA competes with NLS peptide, suggesting that the nuclear import of RHA is mediated by importin α/β pathway [16]. To characterize the nuclear import mechanism of RHA-NLS through importins, we performed the nuclear import assays in digitonin-permeabilized HeLa cells. As a transport substrate, we constructed and purified a fusion protein that comprised RHA-NLS between GST and GFP. To compare the nuclear import pathway of RHA-NLS with those of other known NLSs, T-NLS, and M9 fused to GST-GFP were used as controls. The nuclear import of T-NLS is mediated by importin-α/β and that of M9 is mediated by transportin. As shown in Fig. 3A, RHA-NLS, T-NLS, and M9 substrates all failed to effectively enter the nuclei when incubated in buffer alone (panels a, f, and k). The addition of cytosol as a source of the soluble import factors resulted in efficient nuclear entry of all three substrates (panels b, g, and l). Next, to test the involvement of Ran in the nuclear import of RHA-NLS, we used Ran mutant (G19V Ran) which lacks GTPase hydrolysis activity and inhibits several nuclear import pathways mediated by importin-β family. Addition of G19V Ran-GTP markedly inhibited the nuclear accumulation of all three substrates (panels c, h, and m). These results indicate that the nuclear import of RHA-NLS utilizes Ran-dependent pathway and requires soluble factors. To determine the contribution of nuclear import receptors for the import of RHA, two factors known to block importin-α- and -β-mediated nuclear import pathways were used in the import assays. A synthetic T-NLS peptide chemically coupled to bovine serum albumin (T-BSA) is known to bind directly to importin-α and competitively inhibit the nuclear import of classical NLSs. The N-terminus of importin-α containing IBB domain inhibits importin-β-dependent nuclear import by saturating the importin-α binding site of importin-β. Consistent with

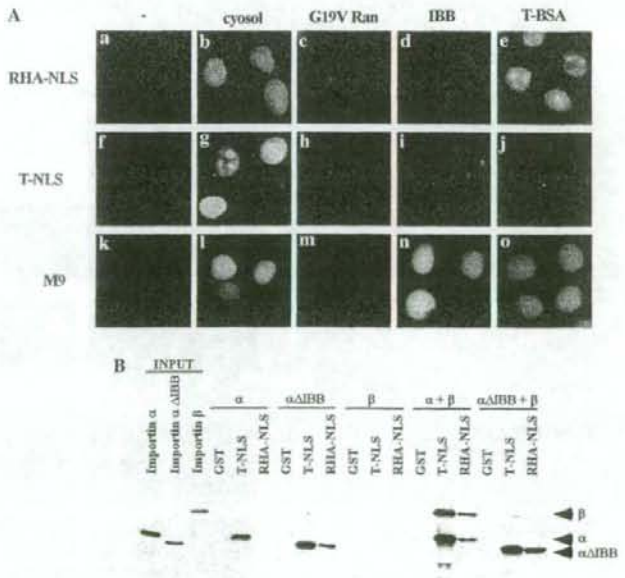

Fig. 3. RHA-NLS is transported into nuclei of digitonin-permeabilized cells. (A) In vitro nuclear import assay. Digitonin-permeabilized HeLa cells were incubated with a reaction mixture containing an ATP regeneration system, each import substrate (GST-T-NLS-GFP, GST-RHA-NLS-GFP, and GSTM9-GFP), and/or indicated effectors, a cytosol (cytosol), cytosol and G19V Ran-GTP (G19V Ran), cytosol and of IBB (IBB), cytosol and of T-BSA (TBSA). (B) Pull-down assay with GST, GST-RHA-NLS or GST-T-NLS-GFP was performed using biotinated importin-α, importin-αΔIBB and importin-β.

previous reports, the nuclear import of T-NLS was markedly inhibited in the presence of excess amount of T-BSA and IBB (panels i and j) while that of M9 was not affected (panels n and o). The nuclear accumulation of RHA-NLS was abrogated by IBB but not by T-BSA (panels e and f). These results suggest that the nuclear import mechanism of RHA-NLS is importin-β-dependent.

To determine whether the RHA-NLS region directly interacts with importin complex, pull down assay was performed. As a probe, GST-NLS expressed in bacterial cells were incubated with biotinated importin-α or -β. As shown as Fig. 3B, RHA-NLS interacted with importin-α/β complex but not with only importin-α alone, whereas T-NLS interacted with importin-α and importin-α/β complex. It is known that the IBB domain has autoinhibitory activity for nuclear import. The IBB domain interacts with its ARM domain which is a binding domain of the NLS of cargo protein. This interaction prevents importin-α to form a complex with cargo proteins and its inhibition is released by interaction of importin-α and -β [36]. To confirm whether the interaction between importin-α and RHA-NLS is inhibited by the autoinhibitory mechanism, importin-α lacking IBB domain (importin-αΔIBB) was used as a probe for pull-down assay. RHA-NLS bound with importin-αΔIBB but not with importin-α. These results suggest that RHA-NLS and importin-α could form a nuclear import complex.

Basic residues in RHA-NLS are important for nuclear import and interaction with importin complex

Although the amino acid sequence of RHA-NLS is unique to RHA homologues, they have basic amino acids as known in the classical NLSs. Previous studies indicated that certain basic amino acids in RHA-NTD are important for the nuclear import of RHA. Mutations were introduced into full-length RHA and the mutants (RHA R1160A, K1163A, and R1166A) were expressed as GFP fusion proteins in HeLa cells (Fig. 4A). The mutant R1160A was localized in the nucleus, whereas K1163A was localized in the cytoplasm. The mutant R1166A was localized in both the nucleus and cytoplasm, although the extent of nuclear accumulation was significantly lower (Fig. 4B). As described in previous studies [16], it is consistent that the two basic residues (K1163 and R1166) are important for the nuclear import activity of RHA-NLS and that K1163 is particularly essential. To test whether the basic amino acids are required for the formation of complex with importin-α and -β, we performed in vitro binding assays using each mutant of RHA-NLS fused to GST. As described previously, the wild type RHA-NLS bound to importin-α and -β. The mutant R1160A interacted with the importin complex similar to the wild type, whereas the mutant R1166A bound weakly and K1163A bound at a level similar to that of GST alone (Fig. 4C). These results emphasize the importance of the basic residues for complex formation of importin-α and β.

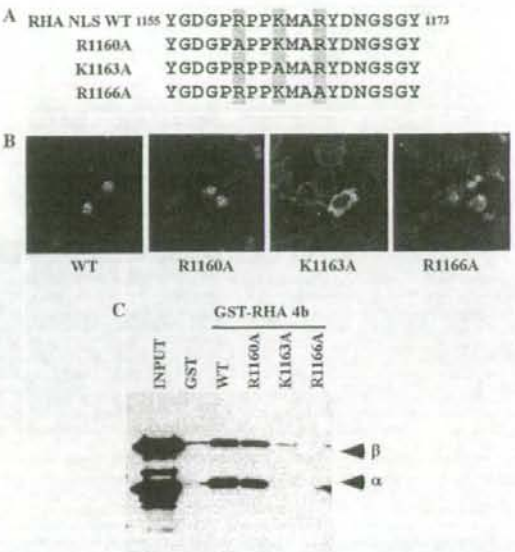

Fig. 4. Alanine substitution of basic residues in RHA-NLS reduces its nuclear migration. (A) Amino acid sequence of RHA-NLS. The mutated three basic residues are indicated by the shaded box. (B) The contribution of basic residues for nuclear import. Full-length RHA and mutant RHAs (R1160A, K1163A, and R1166A) were expressed as GFP-fusion in HeLa cells. (C) Pull-down assay with mutant RHA. RHA 4b with each substitution fused to GST was incubated with biotinated importin-α1 and β.

Importin-α1 and α3 mediate the nuclear import of RHA

Six importin-αs have been identified in human cells, and they are classified into three subfamilies, termed importin-α1, α3, α4, α5, α6, and α7 [27]. We examined the types of importin-α isoforms involved in the nuclear import mechanism of RHA. First, to determine whether RHA-NLS interacts with importin-α1, α3, α4, α5, and α7, pull-down assays were performed using biotinated importin-αs and -β expressed in *E. coli* as probes. Importin-α1, α3, and α4 bound efficiently to RHA-NLS, whereas importin-α5 bound weakly and importin-α7 did not bind (Fig. 5A). The basic residues in RHA-NLS which were important for interaction with importin-α1 had roles for interaction with importin-α3 (data not shown). Next to confirm the specificity of the interaction of RHA-NLS with importin-αs, we carried out in vitro import assays with recombinant transport factors. RHA-NLS and T-NLS substrates were combined with recombinant Ran, importin-β, an ATP-regenerating system, and/or purified importin-αs. As shown in Fig. 5B, incubation without any importin-α failed to induce efficient nuclear import of both substrates. All importin-α isoforms stimulated the nuclear import of T-NLS. For RHA-NLS, importin-α3 showed the best stimulation of the nuclear import and importin-α1 displayed a somewhat weaker effect. Interestingly, importin-α4, α5 or

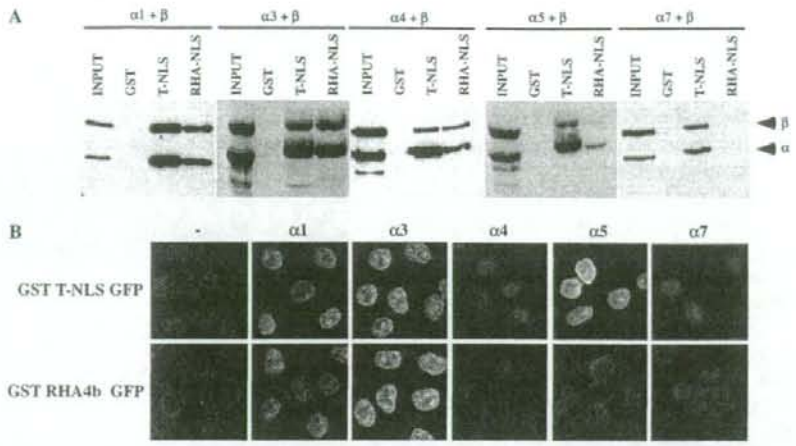

Fig. 5. Qip1 preferentially binds to RHA-NLS. (A) Pull-down assays of recombinant importin isoforms. RHA-NLS fused to GST was incubated with each importin-α, importin-β. (B) In vitro import assay with importin-α isoforms in digitonin-permeabilized HeLa cells.

α7 did not induce nuclear accumulation of RHA-NLS substrate. This was not due to inappropriate preparation of recombinant importin proteins based on the finding that T-NLS was efficiently imported by the addition of these proteins. These results indicate that the nuclear import of RHA is preferentially mediated by importin-α3.

Discussion

In this study, we identified the minimal region of the RHA that is important for its nuclear localization. The NLS of RHA is located at amino acid residue 1155–1173 and consists of 19 amino acids (Fig. 2). RHA formed a nuclear import complex through this region with importin-α and the translocation was mediated by importin-α/β and small GTP-Ran. In this process, importin-α3 was preferentially used for the nuclear import of RHA among six importin-α subfamily members. The 19 amino acid stretch has a few basic residues and the particular residues are important for the interaction with importin complex and the nuclear import of RHA.

It is reported that the NTD comprising 110 amino acids from 1150 to 1250 is essential for the nuclear import and the export of RHA. With regard to nuclear import, it has been indicated that the N terminal half of NTD containing 71 amino acids between 1150 and 1220 is more important, and that the region can transfer into the nucleus [16,28]. The 19 residues identified in the present study are part of the 71 amino acids of NTD and the minimal region for the nuclear import. These results are consistent with previous reports. Furthermore, it is reported that the NTD of RHA is methylated by PRMT1 and the methylation is necessary for the nuclear import mediated by this 110 amino acid stretch [28]. Some proteins, such as heterogeneous nuclear ribonucleoproteins A2 (hnRNP A2) and RNA-binding protein Sam68, are known to have PRMT1-methylated the arginine residues in the RGG domain, and such methylation regulates the translocation [16,37]. However, the relationship between methylation and nuclear import is still not clear. It is thought that the methylation could affect protein interaction. The RHA-NLS region is located next to the RGG domain and does not contain any RGG motifs, although NTD has four RGG motifs. These results suggest that NTD could be divided into two functional regions, the N-terminal for the formation of the import complex and C-terminal for regulation. Methylation in the C-terminal half of NTD may induce conformational change or dissociation from inhibitory factors. These changes may enable the RHA-NLS in the N terminal half of NTD to recruit with importin-α.

The nuclear import of cargo proteins with these classical NLSs utilizes various members of the importin-β families [23]. With regard to RHA-NLS, it has been suggested that the nuclear import is mediated by importin-α/β pathway. Although RHA-NLS directly interacted with importin-α, T-NLS peptide did not compete with RHA-NLS in the nuclear import assay (Fig. 3). While the exact reason for this phenomenon is not clear, several mechanisms could be postulated. One is differences of affinities between NLS and importin-α isoforms. It is reported that importin-α1 and α5 have binding activities for a wide variety of NLS sequences, while importin-α3 exhibits specificity for certain NLSs [38]. Furthermore, it is known that the expression patterns of importin-α subfamilies are tissue-specific and are changed in the differential stages. The binding specificity can be affected by abundance of nuclear import receptors. It is reported that the U1A splicesome protein has two import mechanisms. U1A is translocated through importin-α/β-dependent and cytosol independent pathways. It is suggested that the latter pathway might be negatively regulated under ordinary condition [39,40]. RHA also interacts with various molecules

and has functions in cells. These reports allow us to speculate that nuclear localization mechanisms and the efficiency of the interaction between importin-α and cargo proteins are regulated in the cell type- or signal-dependent manners. These differences in binding specificity may prevent T-NLS from competing with RHA-NLS. The second mechanisms may be related to difference in nuclear import pathway using importin-α/β. NLS interacts with the ARM domains in importin-α. It is known that two T-NLS peptides can bind with each pocket in the ARM domain in vitro binding assay [41]. It is possible that T-NLS and RHA-NLS interact with the different pockets in importin-α. These issues can be addressed by probing the structure of the nuclear import complex.

In the nuclear import process, the two types of classical NLS sequences have been well-analyzed. These sequences are classified into two major groups depending on the numbers of their charged clusters. One is a monopartite sequence with a single consensus motif of basic residues like SV40 T-antigen. The other is a bipartite sequence with two clusters of basic residues with a spacer region like nucleoplasmin. The NLS of RHA which consists of 19 amino acid residues has some basic residues, but it is not consistent with either monopartite or bipartite NLS, basic-basic-X-basic [42]. Comparisons of the amino acid alignment of the NLS sequences show that the RHA-NLS has similarity to that of Ran Binding Protein 3 (RanBP3). Mutational analysis of the NLS of RanBP3 indicated that substitution of the first amino acid in the basic cluster (lysine at amino acid 52) leads to a small nuclear localization and that loss of the double basic residues completely blocked the translocation [43]. In the case of RHA, it has three basic residues and a lysine residue at amino acid 1163 corresponding to lysine at 52 of RanBP3 which is the most important. The next residue of K52 that has only slight effect in RanBP3 is not a basic residue (methionine) in RHA (Fig. 2). RanBP3 NLS also has similarity with c-Myc NLS [43,44]. These results suggest that they could form the same group of NLS.

Our results indicated that the RHA-NLS uses importin-α1 and α3, especially α3 for the nuclear import (Fig. 5). importin-α consists of six isoforms classified into three subgroups. Most cargo proteins, such as SV40 T-antigen, hnRNP K, PCAF [45], and mCRY2 [46], are imported into the nucleus with some efficiency for all importin-α members. On the other hand, some substrate proteins have the specificities for each importin-α isoform. For example, STAT1 [47] and thioredoxin-binding protein 2 (TBP-2) [48] are imported via interaction with importin-α1 or α5. Importin-α3 with which RHA interacts preferentially is also used by other proteins. It was originally isolated as an interactant with DNA helicase Q1/RecQL [49]. STAT3 [50] and RanBP3 [43] have the monopartite of NLS sequences and RCC1 [17,45,51] and mCRY2 [46] have the bipartite NLSs. Their factors are imported into the nucleus using importin-α3. The NLSs of RHA and RanBP3 specifically use importin-α3 for the nuclear import, whereas that of c-Myc does not. The consensus motifs for each importin-α isoforms are unknown. Structural analysis of the cargo proteins could clarify these regulation mechanisms.

Recent studies suggested the involvement of importin-α isoforms in cell-specific functions. Most importins are expressed in several types of tissues except importin-α6 which is limited to the testes [17,25]. The expression levels of importin-α are regulated in cell-specific or signal-dependent manners [52]. For example, importin-α3 is strongly detected in the testes, ovaries and small intestine. Downregulation of importins with small interfering RNAs (siRNAs) revealed that importin-α3 could control cell proliferation and apoptosis [45]. In HL60 cells, the expression levels of each isoform of importin-α are affected during the differentiation stage [52]. Genetic analysis of *Drosophila melanogaster* and *Caenorhabditis elegans* also showed that the germ cells use specific importin isoforms and the individual importins cannot rescue the functions of others [27,53]. For example, *Drosophila* homologue of importin-α3 (Dα3) has a more important role in development, and Dα1 and Dα2 partially rescue the Dα3 [27,54]. However, the relationship between their expression patterns and the selectivity of their cargo proteins for remains to be clarified. RHA is expressed ubiquitously and regulates CREB-dependent transcription [6,8]. The CREB family includes CREB, CREM, and ATF-1 and they are expressed ubiquitously. It is known that CREM has specific functions in the testis. There are tissue-specific splicing isoforms CREM-τ and CREM interacts with testis-specific coactivator [55–57]. These reports suggest that RHA plays important roles as a coactivator of the CREB–CBP complex in the testis and this might be the reason why importin-α3 is used by RHA. We speculate that these may be the underlying mechanisms through which importins regulate the tissue-specific functions of ubiquitously expressed factors. In this study, RHA also bound with importin-α4 as well as importin-α3, which are classified under the same subfamily. Unexpectedly, RHA was not mediated the nuclear localization with importin-α4. It is indicated that some proteins, such as RCC1 and RanBP3 which interact with importin-α3 and α4, are translocated into nuclear by importin-α3 but not by importin-α4 in cells. It is unclear why there are such differences between importin-α3 and α4. They might need the other components for forming the stable import complexes or be competed with factors in cytosol.

In addition to transcription, RHA is involved in the splicing [15] and the export of viral RNA and shuttles between the nuclear and the cytoplasm [17]. It is suggested that RHA does not only export the mRNA but also has functions in the cytoplasm. RHA colocalizes with cytosolic Staufen in the dendrites of differentiated neuroblasts and may regulate the translation [58]. In a preliminary study, we observed that RHA could interact with a ribosomal protein (unpublished data). These findings suggest that the ratio of translocation may regulate the functions

of RHA in cells. RHA uses importin-α1 and α3. The expression level of importin-α3 with which RHA preferentially interacts is stable and are regulated such as housekeeping genes. In contrast, importin-α1 is the most widely expressed in tissues and its levels is regulated by various signals [52]. RHA is predominantly localized in the nucleus and has very important roles in transcription. These might be the reasons for the use of these two importin isoforms (importin-α1 and α3) by RHA-NLS rather than the testis-specific importin-α6. These mechanisms of the nuclear import could allow effective regulation of RHA functions.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.11.161.

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ORIGINAL ARTICLE

High-mobility group box 1 protein promotes development of microvascular thrombosis in rats

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Summary. *Background:* Sepsis is a life-threatening disorder resulting from systemic inflammatory and coagulatory responses to infection. High-mobility group box 1 protein (HMGB1), an abundant intranuclear protein, was recently identified as a potent lethal mediator of sepsis. However, the precise mechanisms by which HMGB1 exerts its lethal effects in sepsis have yet to be confirmed. We recently reported that plasma HMGB1 levels correlated with disseminated intravascular coagulation (DIC) score, indicating that HMGB1 might play an important role in the pathogenesis of DIC. *Objectives:* To investigate the mechanisms responsible for the lethal effects of HMGB1, and more specifically, to explore the effects of HMGB1 on the coagulation system. *Methods:* Rats were exposed to thrombin with or without HMGB1, and a survival analysis, pathologic analyses and blood tests were conducted. The effects of HMGB1 on the coagulation cascade, anticoagulant pathways and surface expression of procoagulant or anticoagulant molecules were examined *in vitro*. *Results:* Compared to thrombin alone, combined administration of thrombin and HMGB1 resulted in excessive fibrin deposition in glomeruli, prolonged plasma clotting times, and increased mortality. *In vitro*, HMGB1 did not affect clotting times, but inhibited the anticoagulant protein C pathway mediated by the thrombin–thrombomodulin complex, and stimulated tissue factor expression on monocytes. *Conclusions:* These findings demonstrate the procoagulant role of HMGB1 *in vivo* and *in vitro*. During sepsis, massive accumulation of HMGB1 in the systemic circulation would promote the development of DIC.

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Introduction

Sepsis is a complex clinical syndrome resulting from systemic inflammatory and coagulatory responses to infection [1,2]. Hyperactivation of the inflammatory system is the most important feature of sepsis, and has been the most common target of therapeutic strategies. So far, diverse therapies directed against proinflammatory mediators have revealed dramatic effects in animal models of sepsis. However, in humans, most of these strategies have not improved survival of septic patients [2]. Coagulopathy is another important feature of sepsis, and 30–50% of patients show the most severe clinical form, called disseminated intravascular coagulation (DIC) [3]. Intravascular fibrin deposition leads to obstruction of the microvascular bed, resulting in development of multiple organ dysfunction syndrome (MODS), including renal insufficiency and acute respiratory distress syndrome [4]. As DIC and MODS are associated with poor outcomes, anticoagulant therapy during sepsis seems to be a valuable option. Activated protein C (APC), an anticoagulant with a broad spectrum of anti-inflammatory effects, led to improved survival in patients with severe sepsis [5]. However, because of bleeding complications, the use of APC therapy needs to be carefully considered in relation to risks and benefits [6]. Thus, it is highly desirable to identify novel targets for the treatment of sepsis.

High-mobility group box 1 protein (HMGB1) is one promising therapeutic target for the treatment of sepsis. Blockade of HMGB1, even at later time points after onset of infection, was shown to rescue mice from lethal sepsis [7,8]. HMGB1 has pleiotropic effects both inside and outside the cell. In the cell nucleus, HMGB1 bends DNA, and promotes protein assembly on DNA targets [9]. When released from necrotic or activated cells, extracellular HMGB1 triggers inflammation, immune responses, and tissue regeneration