Possible mechanism of nuclear translocation of proteasomes

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Proteasomes (multicatalytic proteinase complexes), which are identical to the ubiquitous eukaryotic 20S particles, are localized in both the cytoplasm and the nucleus, but the mechanism of their co-localization in the two compartments is unknown. On examination of the primary structures of subunits of proteasomes, a consensus sequence for nuclear translocation of proteins, X-X-K-K(R)-X-K(R) (where X is any residue), was found to be present in some subunits and to be highly conserved in the subunits of a wide range of eukaryotes. In addition, proteasomal subunits were found to bear a cluster of acidic amino acid residues and also a potential tyrosine phosphorylation site that was located in the same polypeptide chain as the nuclear location signal. These structural properties suggest that two sets of clusters with positive and negative charges serve to regulate the translocation of proteasomes from the cytoplasm to the nucleus, and that phosphorylation of tyrosine in certain subunits may play an additional role in transfer of proteasomes into the nucleus.

Acidic amino acid cluster; Nuclear location signal; Proteasome; 20S particle; Tyrosine phosphorylation site

1. INTRODUCTION

Proteasomes are unusually large (M_r = ~750000) multi-functional enzyme complexes with a highly organized structure composed of many non-identical polysubunits of small size (M_r = 21000–31000) with different charges (pI = 4–9) [1,2]. They have been identified as multicatalytic proteinase complexes with at least three different types of active sites catalyzing cleavage of peptide bonds on the carboxyl side of basic, neutral and acidic amino acid residues of proteins [3]. They are believed to plasy an essential role in an energy-dependent nonlysosomal proteolytic pathway, since their specific antibodies inhibit ATP-, ubiquitin-dependent proteolytic activities almost completely [4,5], and since they are converted to 26S proteolytic complexes by association with certain components and the resulting complexes catalyze ATP-dependent breakdown of ubiquitin-protein conjugates [6,7]. The size, shape and subunit multiplicity of proteasomes have been found to be identical with those of ring-shaped or cylindrical particles of 20S [8,9], which are proposed to be involved in various basically important cellular functions, such as repression of mRNA translation [10] and tRNA processing [11]. Therefore, proteasomal 20S particles are thought to be multifunctional enzyme complexes responsible for the metabolisms of both protein and RNA.

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Proteasomes and related 20S particles are known to be widely distributed in eukaryotes ranging from man to yeast [2,10,12]. They are abundant in various mammalian cells and tissues, constituting about 0.1–1.0% of the total soluble proteins [13]. The apparent half-life of proteasomes in liver was found to be 12–15 days, suggesting their slow turnover [14]. These properties of proteasomes indicate that they belong to a family of proteins with house-keeping function and imply the general importance of their function(s).

In this letter, we summarize data showing that proteasomes are located in both the cytoplasm and the nucleus of a variety of eukaryotic cells. On the basis of data on the primary structures of the subunits of proteasomes, we suggest that proteasomes are translocated into the nucleus by a nuclear signal-mediated transport pathway, and that their translocation may be regulated by interaction with two segments containing an acidic amino acid cluster and a nuclear location signal plus a tyrosine phosphorylation site present in certain subunits.

2. NUCLEAR AND CYTOPLASMIC CO-LOCALIZATION OF PROTEASOMES

Proteasomal 20S particles were first found in the soluble fraction of various tissues, but later also in the nuclei of various eukaryotic cells, as listed in Table I. For instance, the ribonucleoprotein particles named prosomes were first found to be associated with cytoplasmic mRNAs [10], but recently they were also

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Table I

Nuclear and cytoplasmic colocalizations of proteasomes and related 20S particles

Name of particles	Source	Method of detection	Reference
1. Donut-shaped miniparticles	human and rat cells	electron microscopy	[16]
2. 22S cylinder particles	*Xenopus laevis*	immunohistochemical	[17]
3. 5′-pre-tRNAse	*Xenopus laevis* and mammalian cells	biochemical	[11]
4. Prosome	avian erythroleukemic cells	immunocytochemical	[15]
5. Prosome	sea urchin	immunohistochemical	[18]
6. Prosome	axolotl	immunohistochemical	[20]
7. Prosome	newt	immunohistochemical	[21]
8. Multicatalytic protease	sea urchin	biochemical	[19]
9. Proteasome	*Drosophila*	immunohistochemical	[22]
10. Proteasome	rat and human liver	immunochemical and biochemical	[23]
11. Proteasome	rat fibroblasts	immunocytochemical	[9]
12. Proteasome	human leukemic cells	immunohistochemical	[24]
13. Proteasome	human renal cancer cells	immunohistochemical	[25]

found to be present in the nuclei of cells transformed with avian erythroleukemic virus [15]. Donut-shaped 'miniparticles' were found to be present in both the cytoplasm and the nucleus of mammalian cells and to be distributed predominantly in the nuclei of tumor cells [16]. Likewise, the 22S cylinder particles of *Xenopus laevis* were demonstrated in the nucleoplasm as well as the cytoplasm of oocytes and transcriptionally active somatic cells [17]. Pre-tRNA 5′ processing nuclease of *Xenopus laevis* has been purified from the nuclei of oocytes as a highly homogeneous form with a similar structure as the 20S cylinder particles [11]. Interestingly, the distribution of proteasomes in the two subcellular compartments appears to be interchangeable during oogenesis, embryogenesis and development in lower eukaryotic organisms, such as the sea urchin [18,19], axolotl [20], newt [21] and *Drosophila* [22].

Recently, we found that a highly purified nuclear fraction from rat liver contained an appreciable amount of proteasomes with similar proteolytic activity and immunoreactivity to those of proteasomes in the cytosolic fraction and that these proteasomes appeared to be loosely associated with nuclear components in the nucleoplasm [23]. Moreover, immunohistochemical staining of human liver showed that proteasomes were distributed densely in the nucleoplasm and diffusely in the cytoplasm of hepatocytes. About two-thirds of the hepatic nuclei stained strongly with anti-proteasome antibody, whereas the remaining nuclei did not stain, suggesting the rapid interchange of proteasomes between the nucleus and cytoplasm under certain physiological conditions [23]. We also observed that proteasomes were predominantly located in the nuclei of actively dividing rat fibroblasts [9] and various human leukemic and renal cancer cells [24,25]. This variation in the intracellular distribution of proteasomal 20S particles seems important in considering the physiological function of these particles.

3. IDENTIFICATION OF NUCLEAR LOCATION SIGNALS IN PROTEASOMES

Recent studies have shown that translocation of proteins into the nucleus is a multi-step process consisting of recognition and translocation events and that the rapid and selective sorting of nuclear proteins from the majority of other cellular proteins is determined by a structural signal included in nuclear proteins (for a recent review, see [26]). Chemical and genetic-engineering analyses of nuclear proteins and measurement of nuclear import of proteins after their microinjection into living cells have shown that very short regions in the polypeptide chains of proteins are essential for their translocation into the nucleus. The amino acid sequences of these regions are not identical, but all are rich in basic amino acid residues. Very recently, the consensus sequence X-X-**K**-**K(R)**-X-**K(R)**, where X is any residue, was proposed as a nuclear location signal (NLS) [26]. This short signal sequence appears to be sufficient for nuclear translocation of proteins and is highly conserved in a variety of proteins imported into the nucleus, as shown in Table II.

Since proteasomes are present in the nucleus as well as the cytoplasm, as described above, it was important to determine whether proteasomes also contain a similar NLS sequence. Recently, we determined the primary structures of the five main subunits RC2 [27], RC3 [28], RC5 [29], RC8 [30] and RC9 [31] of rat liver proteasomes (consisting of at least thirteen subunits) and two subunits YC1 [32] and YC7-α [32] of yeast proteasomes by recombinant DNA techniques, and found that certain subunits, such as RC3, RC9 and YC7-α have a consensus NLS sequence, X-X-**K**-**K**-X-**K** (Table II), suggesting that proteasomes are transported from the cytoplasm to the nucleus via a nuclear signal-mediated transport pathway. We also determined the primary structures of several subunits of human proteasomes by cDNA sequencing and found that identical NLS sequences are present in the same positions as in the corresponding subunits of rat proteasomes (to be published), indicating that the sequence of the NLS of proteasomes has been highly conserved during evolution. As shown in Fig. 1, the location of the signal sequence differs in different subunits. This difference may be related with the topology of the signal in the tertiary structure of different subunits. Moreover,

Table II

Consensus sequence for nuclear location signals in various proteins and certain proteasome subunits

Nuclear protein or proteasome subunit	Signal sequence
SV40 large-T	126-PK**KKRK**-131
SV40 VP2	318-NK**KKRK**-323
Lamin A	415-VT**KKRK**-420
Histone H2B	29-GK**KRSK**-34
Adenovirus E1A	283-SC**KRPR**-288
Polyomavirus large-T	279-PP**KKAR**-284
	190-SR**KRPR**-195
Human c-myc	321-AA**KRVK**-326
Nucleoplasmin	165-QA**KKKK**-170
N1	547-DA**KKSK**-552
	531-VR**KKRK**-536
Glucorticoid receptor (rat)	512-TK**KKIK**-517
Interleukin-1 receptor	427-MV**KKSR**-432
Cofilin	28-EV**KKRK**-33
Rat RC3	48-TE**KKQK**-53
Rat RC9	249-RE**KKEK**-254
Yeast YC7-α	185-HF**KKSK**-190

Data for various nuclear-imported proteins are quoted from a recent review by Roberts [26], except those for interleukin-1 receptor [36] and cofilin [37]. Data for proteasome subunits RC3, RC9 and YC7-α are from [28], [31] and [32], respectively. The numbers shown are residue numbers of the peptides in the respective proteins

subunits RC3 and RC9 of rat proteasomes were both found to have an NLS motif. So far we have determined the primary structures of only half the subunits. But similar signals may well be present in other subunits whose sequences have not yet been determined because some proteins such as polyoma T-antigen and N1 contain two or more sets of the NLS motif (Table II). Thus, multiple NLS may act cooperatively in nuclear accumulation [26].

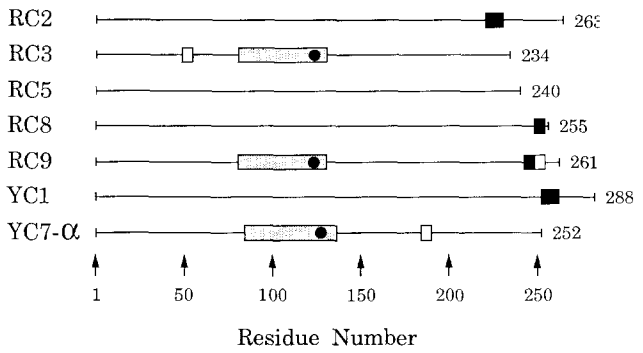

Fig. 1. Schematic representation of the location, of the nuclear location signal, acidic amino acid cluster and region containing the tyrosine phosphorylation site conserved in various proteasome subunits. The open and shaded boxes show the nuclear location signals (X-X-K-K-X-K, where X is any residue) and acidic amino acid cluster, respectively. The dotted boxes show segments homologous to the regions of a family of proteins phosphorylated with tyrosine kinase [34]. The closed circles show the positions of potential tyrosine phosphorylation sites.

Not all the proteasome subunits bear an NLS sequence: for example, the NLS sequence has not been found in subunit C5 (Fig. 1). Moreover, proteasomes in the nuclei are apparently similar in size to those in the cytosol [23]. These findings suggest that proteasomes traverse the nuclear membranes as intact complexes, not as free subunits. Proteasome complexes may be too large to be transported through nuclear pores, but the nuclear signal-mediated transport pathway is known to allow large nuclear proteins to enter the nucleus rapidly: for example, ribosomes, which are much larger particles, freely traverse the nuclear pores [26].

4. ACIDIC AMINO ACID CLUSTERS AND TYROSINE PHOSPHORYLATION SITE

Recently, Yoneda et al. [33] suggested that the binding pocket of the putative nuclear signal receptor in nuclear pores contains numerous acidic residues that interact in a complementary fashion with the basic residues of the NLS sequence. They raised an antibody against a synthetic peptide, D-D-D-E-D, which is complementary to the NLS sequence of the SV40 large-T signal peptide and found that on microinjection into cultured cells, this anti-peptide antibody bound to nuclear envelopes and blocked NLS-mediated uptake of proteins into the nucleus.

On determination of the primary structures of proteasomes, we found that several proteasome subunits bear an acidic amino acid cluster that is enriched in glutamyl and aspartyl residues. These acidic amino acid clusters are idential, or very similar to the D-D-D-E-D sequence thought to be included in the putative nuclear receptor (Table III), suggesting that this cluster of negatively charged amino acids may interfere with the interaction between proteasomes with a positively charged NLS and nuclear receptor protein with a negatively charged cluster in nuclear pores. These negatively charged clusters were found in the carboxyl-terminal regions of RC2, RC8, RC9 and YC1 (Fig. 1), indicating the functional importance of their location, such as at the surface of molecules. Moreover, these segments in proteasomes appear to have been conserv-

Table III

Clusters of acidic amino acid residues in some proteasome subunits

Subunit	Sequence
YC1	250-**DDDEDEDDSD**-259
RC2	218-**DLEFTIYDDDD**-228
RC8	246-**EEDESDDD**-253
RC9	241-**EEEEAKAERE**-250

The numbers shown are residue numbers of clusters in the respective proteins. The sequence data for YC1, RC2, RC8 and RC9 are taken from Fujiwara et al. [32], Fujiwara et al. [27], Tanaka et al. [30] and Kumatori et al. [31], respectively

ed during evolution of eukaryotes ranging from yeast to mammals.

As shown in Fig. 2, computer-assisted homology analysis revealed that a sequence of about 50 amino acid residues in components RC3 and RC9 of rat proteasomes closely resembles a conserved region with a possible autophosphorylation site found in viral and receptor proteins with tyrosine kinase activity, such as $pp60^{c\text{-}src}$, a cellular protein with homology to the oncogene product of Rous avian sarcoma virus, and receptors of epidermal growth factor and insulin [34]. A similar tyrosine phosphorylation site was also conserved in a corresponding region of subunit YC7-α of yeast proteasomes (Fig. 2). Thus, this region may be generally important for expressing proteasome functions. In fact, several subunits of *Drosophila* proteasomes were demonstrated to be phosphorylated in certain stages of development [35].

Interestingly, on determination of the primary structures of proteasomes, we found that these potential tyrosine phosphorylation sites are located in the same polypeptide chain as the NLS sequence [28,32]. As shown in Fig. 1, the two sites coexisted in subunits such as RC3, RC9 and YC7-α. Thus, tyrosine phosphorylation of these proteasomal subunits may affect the NLS function, that is, the nuclear translocation of proteasomes.

5. POSSIBLE REGULATORY MECHANISM FOR TRANSLOCATION OF PROTEASOMES FROM THE CYTOPLASM TO THE NUCLEUS

As discussed above, proteasomes have an NLS segment and may be translocated from the cytoplasm to the nucleus through a nuclear signal-mediated pathway. However, nuclear translocation of proteasomes appears to be somewhat different from that of typical nuclear proteins, because proteasomes are present in both the cytosol and the nucleus, whereas most known nuclear proteins are present only in the nucleus. Therefore, there must be some mechanism that regulates nuclear translocation of proteasomes.

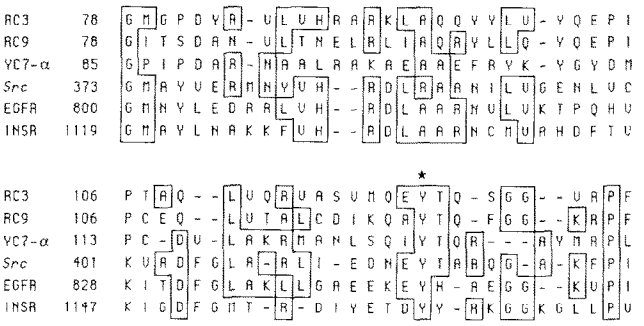

Fig. 2. Comparison of parts of the amino acid sequences of RC3, RC9, YC7-α and autophosphorylation regions of a family of proteins with tyrosine kinase activity. Residues that are identical in rat proteasome RC3 and RC9, yeast proteasome YC7-α and other gene products are boxed. The numbers shown are the residue numbers of the respective proteins. Gaps (bars) are inserted for optimal alignment of the sequences. The asterisk indicates the autophosphorylation tyrosine residue conserved in this family of tyrosine kinases. The sequence data for Src ($pp60^{c\text{-}src}$), a cellular protein with homology to the oncogene product from Rous avian sarcoma virus, human epidermal growth factor receptor (EGFR) and human insulin receptor (INSR) are from Hanks et al. [34].

The glucocorticoid receptor is normally present in the cytosol, but is transported into the nucleus when it is associated with glucocorticoid [26]. Probably the NLS of a glucocorticoid receptor in the cytosol is masked by its association with HSP90 protein, but once glucocorticoid binds to its receptor, the ligand-receptor complex releases HSP protein and undergoes a conformational change that results in its transfer into the nucleus. The actin molecule itself does not bear a NLS sequence, but actin is known to be transported into the nucleus in response to heat-shock stress. This is due to its association with cofilin containing a NLS segment [37].

Based on these findings, we propose the possible mechanism for regulation of nuclear translocation of proteasomes shown schematically in Fig. 3. The cluster of acidic amino acid residues in proteasomes is assumed to antagonize the nuclear transport mediated by the NLS segments. We assumed that there are two types of proteasomes with different conformations: One type is a cytoplasmic form in which the NLS is buried in the interior of the molecule or masked by the acidic cluster, resulting in suppression of binding of proteasomes to the nuclear receptor in nuclear pores. The other type is the form imported into nuclei in which the positive NLS domain is exposed on the surface of the molecule or released from interaction with the acidic cluster, with consequent acquisition of an active signal for nuclear translocation. This type of proteasomes readily associates with the putative receptor of nuclear proteins by complementary charge interaction, which is the initial event in translocation of proteins into the nucleus and their rapid traverse of the nuclear membrane.

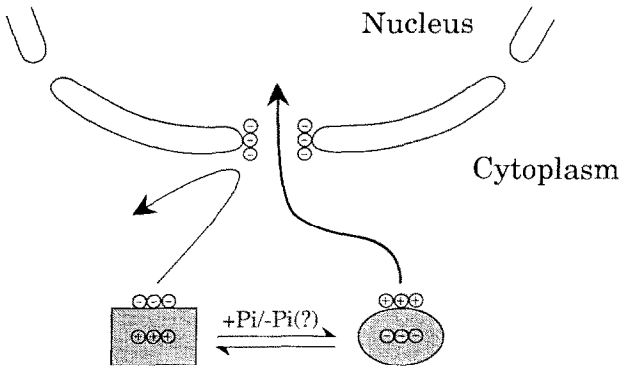

Fig. 3. Schematic representation of a possible mechanism for nuclear translocation of proteasomes. For details, see text. +Pi, phosphorylation; −Pi, dephosphorylation. + and −, amino acid residues with positive and negative charges, respectively.

Thus, the distribution of nuclear and cytoplasmic proteasomes may be determined by the balance of these two forms of proteasomes.

In this scheme, an important problem is how the levels of these two forms of proteasomes are regulated. In this connection it is noteworthy that both the NLS segment and the potential tyrosine phosphorylation site are located in the same polypeptide chain. Interestingly, the cytoskeleton-associated 350-kDa proteins are reported to be phosphorylated and then transferred to the nucleus on mitogenic stimulation of quiescent fibroblasts with serum or growth factors [38]. Thus tyrosine phosphorylation of certain proteasome subunits may affect the NLS function of these subunits by changing their conformation or altering their subunit organization to expose the NLS segment on the surface of the complexes: in other words, conversion of the cytoplasmic to nuclear-directed forms may be regulated by tyrosine phosphorylation. Tyrosine phosphorylation is known to act as an intracellular second-messenger for transduction of external signals stimulated by various hormones and growth factors. Thus, this phosphorylation may serve to initiate inducible or selective translocation of proteasomes. This phosphorylation site is conserved not only in subunits RC3 and RC9 of rat proteasomes but also in subunit YC7-α of yeast proteasomes, indicating its common function in proteasomes.

5. CONCLUSIONS

As discussed in this paper, we found by sequence analyses that several subunits of eukaryotic proteasomes bear a possible consensus sequence for nuclear protein location, that a unique cluster of acidic amino acid residues is present in the carboxyl-terminal regions of some proteasome subunits, and that a potential tyrosine phosphorylation site is present in the polypeptide subunits bearing the NLS sequence. From these results, together with reported findings, we propose that the balance of the nuclear and cytoplasmic distributions of proteasomes may be regulated by complementary or antagonistic charge-interactions of the two sets of positively and negatively charged clusters, and that tyrosine phosphorylation of certain proteasome subunits may affect the function of the nuclear location signal present in the same polypeptide chain. Thus, the nuclear location signal, acidic amino acid cluster and tyrosine phosphorylation site may play important roles in nuclear translocation of proteasomes, which may be responsible for their specific functions in the nucleus and cytoplasm.

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