Identification of Nuclear Export Signals in Antizyme-1*

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Antizyme-1 (AZ1) is a protein that negatively regulates polyamine synthesis by inhibiting the key synthetic enzyme ornithine decarboxylase and targeting it for degradation by the 26 S proteasome. Recent work shows that antizyme protein translocates to the nucleus during mouse development (Gritli-Linde, A., Nilssom, J., Bohlooly, Y. M., Heby, O., and Linde, A. (2001) Dev. Dyn. 220, 259–275). However, the significance and mechanism of this phenomenon remain unclear. In this study, we expressed AZ1 fused with enhanced green fluorescent protein (EGFP) to study its localization in a living cell. We found that EGFP-AZ1 was predominantly localized in the cytoplasm and that treatment with leptomycin B, a specific inhibitor of chromosomal region maintenance 1 (CRM1) induced nuclear accumulation of EGFP-AZ1 in Chinese hamster ovary and NIH3T3 cells. Two independent nuclear export signal (NES) sequences, each containing essential hydrophobic residues, were identified in the 50 N-terminal amino acid residues and in the central part of AZ1. The activity of the second NES was inhibited by an N-terminal adjacent region and was only revealed in N-terminal truncated constructs. Both NESs were active when fused to an artificial nuclear protein SV40-NLS-EGFP-EGFP. The ability of AZ1 to shuttle between the nucleus and the cytoplasm suggests that it has a novel function in the nucleus.

Polymamines are ubiquitous organic cations with tightly regulated cellular concentrations and are essential for cell growth and development (1, 2). Antizyme (AZ)† was first described as an inhibitor of ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis (3). When cellular polyamine levels rise, AZ is induced and then binds to the monomeric form of ODC (4, 5), which makes the enzyme a preferential substrate for proteolysis by the 26 S proteasome without ubiquitination (6). AZ also inhibits the uptake of extracellular polymamines (7, 8). There are at least three independent antizyme isoforms conserved among mammals. AZ1 and AZ2 have a wide tissue distribution, whereas AZ3 is testis-specific (9, 10). Expression of AZs is induced by a unique translational frameshift mechanism (11). For AZ1, when cellular polyamine levels are low, the polypeptide corresponding to amino acid residues 1–68 is produced and translation is terminated at the following UGA codon (11). When cellular polyamine levels are high, +1 frameshifting occurs after the 68th codon is decoded, resulting in the full-length antizyme protein (amino acid residues 1–227). All of the known regulatory activities of AZ1 are present in amino acid residues 69–227 (12). A second AUG exists in AZ1 mRNA at the 34th codon, and this second AUG is also utilized as initiation codon (11, 13). The two resulting translation products have different half-lives (14). Furthermore, it has been suggested that the N-terminal region of the translation product from the first AUG contains a putative mitochondrial targeting signal and thus the distribution of AZ1 may depend on this region (15). It is currently unclear whether the short polypeptides (residues 1–68 and 34–68) exert their specific functions.

Gritli-Linde et al. (16) recently revealed nuclear localization of AZ and ODC during mouse development using systematic expression analysis at the mRNA and protein levels. Gruender et al. (17) reported that the transcription factor Smad1 interacts with both AZ1 and proteasome subunit HsN3 and that this ternary complex enters the nucleus in response to bone morphogenetic protein type I receptor activation (18). These findings have led to speculation that a specific mechanism is responsible for shuttling AZ1 between the nucleus and the cytoplasm. Here we show the subcellular localization of AZ1 in living cells using fusion proteins of enhanced green fluorescent protein (EGFP) and AZ1. We found that in the presence of leptomycin B (LMB), an inhibitor for chromosomal region maintenance 1 (CRM1), also known as exportin-1-dependent nuclear export (19, 20), AZ1 that fused to EGFP (EGFP-AZ1) accumulates in the nucleus, whereas in the absence of LMB, EGFP-AZ1 remains primarily in the cytoplasm. We then used deletion analysis and site-directed mutagenesis to identify the nuclear export signal (NES) sequences in AZ1.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-directed Mutagenesis—To generate an AZ1 construct fused with EGFP at the N terminus, an Xhol/EcoRI DNA fragment encoding EGFP and an EcoRl/XbaI DNA fragment encoding an in-frame mutant form of rat AZ1 (AZ1AT) were amplified by PCR from pd2EGFP-N1 vector (Clontech) and pGEM4Z/Δ205 (11), respectively. These fragments were inserted in tandem into a pd2EGFP-N1 vector (Clontech) at the XhoI and XbaI restriction sites, resulting in pEGFP-AZ1. Deletion mutants were prepared by replacing the EcoRl/XbaI fragment of pEGFP-AZ1 with partial EcoRl/XbaI fragments of AZ1AT amplified by PCR. To generate plasmid pCMV-Myc-AZ1, an EcoRI/KpnI DNA fragment encoding AZ1AT was amplified as above and inserted into the EcoRI/KpnI sites of the pCMV-Myc vector (Clontech).

To generate pEGFP-EGFP vectors, two EGFP fragments (XhoI/HindIII and HindIII/EcoRI) were amplified by PCR and the EcoRl/XbaI fragments of AZ1AT and their mutants were inserted into pd2EGFP-N1. A synthetic oligonucleotide corresponding to SV40 large T-antigen NLS (PKKKKRK) (21) was fused to the N terminus of pEGFP-EGFP-AZ1 using BglII/HindIII sites (pNLS-EFGP-EGFP-AZ1). Drosophila AZAT (D-AZAT) cDNA was provided to us by Drs. J. F. Atkins and I. P. Ivanov. pEGFP-D-AZ was prepared by replacing the EcoRl/XbaI cDNA

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‡ The abbreviations used are: AZ, antizyme; EGFP, enhanced green fluorescent protein; LMB, leptomycin B; ODC, ornithine decarboxylase; CHO, Chinese hamster ovary; CRM1, chromosomal region maintenance 1; D-AZ, Drosophila AZ, NES, nuclear export signal; NLS, nuclear localization signal; MTS, mitochondrion targeting signal.

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fragment of AZ1 in pEGFP-AZ1 with an EcoRI/XhoI fragment of D-AZ1AT amplified by PCR.

Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) to generate a series of mutations in the NLS fragments. The wild-type NLS was changed to PAKKRRKV (m1NLS), PKKAKRRKV (m2NLS), or PAKKRKRKV (m3NLS). All of the constructs were verified by sequencing with an ABI PRISM 3700 sequencer and Big Dye® terminator cycle-sequencing FS reaction kit (ABI).

Cell Culture—Mouse NIH3T3 cells were maintained in Dulbecco's modified Eagle medium containing 10% calf serum. Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle medium containing 4% newborn calf serum and 2% fetal calf serum. All of the cells were grown at 37°C in a humidified 5% CO2 atmosphere.

Transfection and LMB Treatment—Plasmid DNA for transfection was purified using a Plasmid Miniprep Kit (Qiagen) according to the manufacturer's instructions. Cells were seeded onto 35-mm sterile polystyrene dishes or glass bottom dishes (IWAKI, Tokyo, Japan) and transiently transfected at 50–70% confluency with 1 μg of plasmid DNA using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. At 24 h post-transfection, cells were washed with fresh growth medium and processed for fluorescence microscopy. When indicated, LMB was added to the culture medium at a final concentration of 5 ng/ml for 2.5 h.

Fluorescent Microscopy—EGFP fusion proteins in living cells were visualized using an Olympus IX70 inverted fluorescence microscope equipped with a Polaroid PDMC 1e digital camera. Nuclear DNA was stained with 100 ng/ml Hoechst 33342 (Molecular Probes) for 30 min under culture conditions. After staining, cells were washed twice with fresh medium and incubated for 1 h before observation. For immunocytochemical detection of Myc-tagged protein, cells were seeded on glass-bottom plates for transfection. After expression, cells were fixed in 4% paraformaldehyde at 4°C for 10 min. Aldehyde groups were quenched by incubating the cells with 10 mM glycine (pH 8.5) at room temperature for 5 min. Fixed cells were incubated with 3% pre-immune goat serum in phosphate-buffered saline at room temperature for 1 h then with anti-Myc monoclonal antibody (Medical & Biological Laboratories) diluted 1:500 in blocking solution (phosphate-buffered saline containing 3% pre-immune goat serum) at room temperature for 1 h or at 4°C overnight. Myc-tagged protein was detected using monoclonal anti-IgG antibodies conjugated with Alexa Fluor-545 fluorescence probes (Molecular Probes).

RESULTS

Subcellular Distribution of EGFP-AZ1 and Its Nuclear Accumulation with LMB—To examine the subcellular localization of AZ1 in living cells, NIH3T3 and CHO cells were transiently transfected with expression vectors of EGFP-AZ1 or EGFP alone and analyzed by a fluorescence microscope. Approximately 60% of the cells expressed EGFP-AZ1, and 80–90% of these cells exhibited the fluorescence localized primarily in the cytoplasm in the absence of LMB, a specific inhibitor of CRM1-dependent nuclear export (Fig. 1, −LMB) (19, 20). The addition of LMB-induced nuclear accumulation of EGFP-AZ1 in both cell lines (Fig. 1). This was observed in at least 70% EGFP-expressing cells (Fig. 3B). In almost all of the cells transfected with non-fused EGFP, fluorescence was distributed in both the cytoplasm and the nucleus, irrespective of LMB treatment (Fig. 1). To confirm the subcellular localization of AZ1 not fused with EGFP but tagged with a small epitope, NIH3T3 cells were transfected with vectors expressing Myc-tagged AZ1 (Myc-AZ1) and examined immunohistochemically. As shown in Fig. 2, Myc-AZ1 was localized predominantly in the cytoplasm and LMB treatment induced accumulation of Myc-AZ1 in the nucleus. Western blot analysis of whole cell extracts followed by immunostaining with monoclonal anti-EGFP or anti-Myc antibodies demonstrated that EGFP-AZ1 and Myc-AZ1 proteins were expressed in the cells and that no proteolytic fragments containing EGFP were present (data not shown). These results indicate that AZ1 protein is subject to shuttling between the nucleus and the cytoplasm and that nuclear export is mediated by a CRM1-dependent pathway (19, 22).

AZ1 Contains Two Nuclear Export Signals—We attempted to determine which region is important for the nucleocytoplasmic shuttling of AZ1. We first divided AZ1 into the N-terminal portion (amino acid residues 1–68) and the remaining portion (amino acid residues 69–227). These portions were fused with EGFP and introduced into cultured cells. In CHO cells, the fusion protein containing the N-terminal portion (EGFP-AZ11–68) was localized mainly in the cytoplasm in the absence of LMB (Fig. 3A). LMB treatment prevented the exclusion of EGFP-AZ11–68 from the nucleus, resulting in an even fluorescence distribution between the cytoplasm and the nucleus. EGFP-AZ169–227 was localized in the cytoplasm and the nucleus almost evenly in the absence of LMB. LMB induced nuclear accumulation of EGFP AZ169–227 (Fig. 3A). The results of quantitative analysis of at least 200 fluorescent-positive cells are shown in Fig. 3B. >70% of cells showed cytoplasmic distribution of EGFP.
AZ1Δ1–68 in the absence of LMB. LMB caused a shift to nucleocytoplasmic or even nuclear distribution. LMB also induced nuclear accumulation of EGFP-AZ1Δ69–227 in >50% of cells. These results suggest that AZ1 contains two NES sequences.

N-terminal NES—EGFP-AZ1Δ69–227 as well as mutants with smaller N-terminal deletions, such as EGFP-AZ1Δ10–227 and EGFP-AZ1Δ34–227, were evenly distributed in the cytoplasm and the nucleus in the absence of LMB (Fig. 4A). All of the C-terminal deletion mutants containing the first 50 amino acid residues, such as EGFP-AZ1Δ11–134, EGFP-AZ1Δ61–68, EGFP-AZ1Δ1–64, EGFP-AZ1Δ1–60, EGFP-AZ1Δ58, and EGFP-AZ1Δ50, were primarily distributed in the cytoplasm in the absence of LMB and became evenly distributed in the cytoplasm and the nucleus after LMB treatment (Fig. 4B). In contrast, mutants with further deletions from the C terminus, such as EGFP-AZ1Δ1–45, EGFP-AZ1Δ40, EGFP-AZ1Δ33, and EGFP-AZ1Δ20, were distributed in the cytoplasm and the nucleus irrespective of LMB treatment (Fig. 4B). Thus, truncation of only nine amino acids from the N terminus of AZ1 resulted in diffuse distribution of the fusion protein, whereas 50 N-terminal amino acid residues were necessary for cytoplasmic distribution on the EGFP fusion protein. Therefore, we refer to amino acid residues 1–50 of AZ1 as NES1.

It has been reported that CRM1 binds to cargo proteins at the leucine-rich NES region (20). The first 10 residues of AZ1 are conserved among vertebrates, and positions of hydrophobic residues are similar to previously identified leucine-rich NES sequences (Fig. 5). To investigate the contribution of these hydrophobic residues, we generated another series of mutant constructs by replacing the hydrophobic residues of NES1 with alanine (Fig. 6, A and B). When substitutions were introduced into EGFP-AZ1Δ68, NES activity was affected to varying degrees (Fig. 6A). L6A and V2A resulted in diffuse distribution in the majority of cells. A combination of I9A/L10A or L28A/L36A/L37A, however, changed subcellular distribution only slightly. The same sets of mutations were also tested in the full-length constructs (EGFP-AZ1Δ227). In this context, only the L6A mutation, either alone or in combination with other mutations, resulted in the disappearance of cytoplasmic distribution. Therefore, Leu-6 appears to be the most important residue for NES1 function. The difference in the results for EGFP-AZ1Δ68 and EGFP-AZ1Δ227 may be due to partial activity of the second NES as discussed below.

Second NES—In contrast to N-terminal truncated mutants losing nuclear exclusion, two longer truncations, namely EGFP-AZ1Δ110–227 and EGFP-AZ1Δ134–227, exhibited nuclear exclusion (Fig. 4A). In the absence of LMB, these fusion proteins were localized in the cytoplasm almost exclusively. Furthermore, shorter AZ1 regions containing residues 114–134 exhibited cytoplasmic distribution when fused with EGFP (EGFP-AZ1Δ110–150 and EGFP-AZ1Δ114–134) in Fig. 4A) in the absence of LMB. For these constructs, LMB treatment induced diffuse distribution. This finding suggests that amino acid residues 114–134 contain a second NES (NES2) and that the N-adjacent region (residue 69–109) carries a regulatory element to NES2. The NES2 region also contains hydrophobic amino acid residues that are conserved among vertebrates and were present in the Drosophila sequence but show little similarity with the consensus sequences of the previously identified NES (Fig. 5). The contribution of the hydrophobic residues to NES2 activity was tested in EGFP-AZ1Δ134–227 as described for NES1. Mutation at one of or two hydrophobic residues, L116A, I118A, L122A, and L116A/L118A, had no effect on nuclear export. However, a combination of three mutations, L116A/I118A/L122A, or four mutations, L116A/I118A/T121A/L122A, abolished nuclear exclusion (Fig. 7A). Therefore, the hydrophobic residues also appear to be important for NES2 activity. The same combinations of mutations were introduced next to the full-length EGFP-AZ1Δ1–227. Both EGFP-AZ1Δ1–227 (L116A/I118A/L122A) and EGFP-AZ1Δ1–227 (L116A/I118A/T121A/L122A) were distributed mainly in the cytoplasm (Fig. 7B). Observation of >200 cells revealed that the distribution of this mutant was identical to that of EGFP-AZ1 (wild type) both in the presence and absence of LMB (data not shown). This finding is consistent with the presence of a functional NES1 region in these mutants. For further testing of NES2 in the absence of NES1, we introduced substitutional mutations into both NES1 (L6A) and NES2 (L116A/I118A/L122A) regions of EGFP-AZ1Δ227. However, the fusion products formed aggregates in the cells and results were inconclusive.

NES1 and NES2 Exhibit Activity to Export a Large Karyophilic Reporter Protein—The results thus far suggest that AZ1 shuttles between the nucleus and the cytoplasm and that two NESs are responsible for this extranuclear transport. However, some of the constructs examined were small enough to diffuse
freely through the nuclear envelope pores. Therefore, we constructed larger reporter proteins composed of two EGFPs connected in tandem ((EGFP)$_2$). CHO cells were transfected with (EGFP)$_2$ full-length AZ1 fusion constructs. (EGFP)$_2$-AZ1 fusion protein was distributed in the cytoplasm in 80% LMB-un-treated cells and both in the nucleus and the cytoplasm evenly in >90% LMB-treated cells (data not shown). To better investigate the nuclear export activity of AZ1 NESs, we introduced next a NLS from SV40 T-antigen and its mutant sequences at the N terminus of the (EGFP)$_2$ reporter. The mutants of the SV40-NLS bear one (m1NLS and m2NLS) or two (m3NLS) lysine to alanine substitutions and are expected to have diminished NLS activity (Fig. 8A). As shown in Fig. 8B, (EGFP)$_2$-AZ1 with the wild-type SV40 NLS was found predominantly in the nucleus and two mutants, m2NLS-(EGFP)$_2$-AZ1 and m3NLS-(EGFP)$_2$-AZ1 in the cytoplasm, in the absence of LMB. The other mutant, m1NLS-(EGFP)$_2$-AZ1, showed an intermediate distribution. In the presence of LMB, the distribution of the fusion proteins was shifted toward the nuclear direction for all of the constructs. These results demonstrate that nuclear import with the wild-type SV40-NLS seems to outbalance the export by the NESs of AZ1 and that m2NLS directs a desirable level of nuclear localization for measuring the activities of AZ1 NESs. We then measured the distribution of m2NLS-(EGFP)$_2$ fusion protein with several mutants of AZ1. m2NLS-(EGFP)$_2$-AZ1$_{1-50}$, which carries the NES1 region, and m2NLS-(EGFP)$_2$-AZ1$_{114-227}$, which carries NES2 region, were observed predominantly in the cytoplasm as m2NLS-(EGFP)$_2$ full-length AZ1 in the absence of LMB and showed diffuse or nuclear distribution in the presence of LMB. On the other hand, two constructs carrying mutations in NES1 (m2NLS-(EGFP)$_2$-AZ1$_{135-227}$) or NES2 (m2NLS-(EGFP)$_2$-AZ1$_{114-227}$), which carries NES2 region, were observed predominantly in the cytoplasm as m2NLS-(EGFP)$_2$ full-length AZ1 in the absence of LMB and showed diffuse or nuclear distribution in the presence of LMB. The other mutant, m1NLS-(EGFP)$_2$-AZ1$_{114-227}$, which carries NES2 region, were observed predominantly in the cytoplasm as m2NLS-(EGFP)$_2$ full-length AZ1 in the absence of LMB and showed diffuse or nuclear distribution in the presence of LMB. These results indicate that both NES1 and NES2 are functional when fused with a reporter protein that is 1) sufficiently large to avoid entering or exiting the nucleus by means of diffusion and 2) localized in the nucleus as a result of the attached NLS. In addition, this confirms the previous conclusions with the simple EGFP reporter that NES1, particularly Leu-6, is necessary for nuclear export of the full-length AZ1 and that either NES1 or NES2 is sufficient when attached to a reporter.

**NES1 Is Not Conserved in Drosophila AZ**—As shown in Fig.
The N-terminal 12 amino acid residues of AZ1 are identical among all of the vertebrates and this region contains a cluster of hydrophobic residues sharing a similarity to known NES sequences. However, this region is not conserved in Drosophila AZ (D-AZ). On the other hand, the hydrophobic residues in the NES2 region (amino acids 114–134) are conserved among ver-
tebrates as well as Drosophila. We investigated subcellular distribution of fusion protein of EGFP and the full-length D-AZ in CHO cells. As shown in Fig. 9, A and B, EGFP-D-AZ showed either nucleocytoplasmic or nuclear distribution and treatment with LMB promoted nuclear accumulation. This pattern is very similar to those seen for the AZ1 mutants having small N-terminal truncations (EGFP-AZ110–227 and EGFP-AZ134–227 in Fig. 4A). Because the CRM1-mediated nuclear export system is conserved between mammals and Drosophila (26), it is probable that NES1 is not present in D-AZ. It is also suggested that the NES2-like region of D-AZ is functional, but its activity is diminished in the full-length peptide.

**DISCUSSION**

**Characterization of AZ1 NES Regions**—We demonstrated that AZ1 protein shuttles between the nucleus and cytoplasm in mammalian cultured cells using LMB, an inhibitor of CRM1-dependent nuclear export. Two NES regions, one at the N

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**Fig. 8.** Localization of fusion proteins containing AZ-NES and SV40 T-antigen NLS or its mutants. A, structures of NLS-EGFP-EGFP-AZ1 fusion protein and mutants. B, quantitative analysis of distribution of fusion proteins described in A. Cellular localization of each fusion protein was determined as for Fig. 3.
terminus (NES1) and the other in the central region of AZ1 (NES2), were identified.

In the N-terminal region, 10 amino acid residues are conserved among vertebrates and the position of the hydrophobic residues match the known consensus sequences of CRM-dependent NESs (Fig. 5). Deletion of this region or mutation of a single hydrophobic residue (leucine 6 to alanine) abolished the cytosolic distribution of the fusion protein (Fig. 6A). Attachment of this 10 amino acid region to EGFP, however, was not sufficient to result in nuclear exclusion. A 50-residue N-terminal segment was necessary (Fig. 4B). Mutations of hydrophobic residues within residues 11–50 did not affect nuclear export (Fig. 6A and B). The necessity of an extended segment for NES function is not typical of leucine-rich NESs (19, 27) but is similar to the NES of mitogen-activated protein kinase-activated protein kinase 2 (28).

NES2 is located within amino acid residues 114–134 where a cluster of hydrophobic residues also exists but does not share any similarities with known NESs. However, the sequence is conserved not only among vertebrates but also in Drosophila (Fig. 5). Point mutational analysis revealed that the hydrophobic residues within this segment, such as Leu-116, Ile-118, and Leu-122, are also important for its function (Fig. 7A). A striking feature of this signal is that it is not evident in full-length AZ1 (Fig. 7B). NES2 activity was only demonstrated clearly in 5’-truncation constructs, such as EGFP-AZ110–227, and EGFP-AZ1114–227, or constructs with a short segment, such as AZ1110–150 and AZ1114–134 (Fig. 4A and 8B). It is most likely that the N-terminal adjacent region (residues 69–109) contains an element(s) that masks or counteracts NES2 activity. However, a closer look at the effects of LMB on the distribution pattern of NES1 mutants such as EGFP-AZ110–227, EGFP-AZ1134–227 (Fig. 4A), and m2NLS-(EGFP)2-AZ1L6A (Fig. 8B) as well as NES1-lacking Drosophila AZ fused with EGFP (Fig. 9) indicate that NES2 is partially active in the presence of the putative negative element. Interestingly, this putative negative element does not inhibit NES1 (compare AZ11–68 and AZ111–134 in Fig. 4B). We attempted to further characterize the putative negative element with mutational analysis, but some of the key constructs such as EGFP-AZ1135–134, EGFP-AZ110–110, and EGFP-AZ115A116A118A122A merely aggregated in the cells; thus, the results were inconclusive.

Multiple Forms of AZ1 and NESs—There are two possible initiator AUG codons in the coding region of AZ1 mRNA. It has been shown that the second AUG at codon 34 is more often used but the translation product from first AUG codon (the full-length product) is also present in vivo. Based on our observations, it is clear that the full-length AZ1 will be effectively exported from the nucleus. The product from the second AUG may be excluded from the nucleus but only weakly as it contains NES2 and the putative negative element but not NES1. It is also possible that NES2 is usually masked, but interaction between AZ1 and another protein induces a conformational change of AZ1 to activate NES2. Alternatively, NES2 may function in shorter variants of AZ1 generated by limited proteolysis (28).

How Does AZ1 Enter the Nucleus?—Nucleocytoplasmic shuttling proteins can enter the nucleus either by simple diffusion or by active transport mechanisms. The nuclear pore complex (NPC) creates aqueous channels ~9 nm in diameter, which allow the diffusion of small proteins with relative molecular masses of 40–60 kDa (29, 30). EGFP-AZ1 has a molecular mass of ~54 kDa and can probably enter the nucleus by simple diffusion. However, the strong nuclear accumulation of EGFP fused with full-length AZ1 was observed in the presence of LMB, suggesting that AZ1 probably includes an NLS. In the presence of LMB, N-terminal deletions up to residue 113 predominantly accumulated in the nucleus. In contrast, EGFP-AZ1135–227 was evenly distributed both in the cytoplasm and the nucleus (Fig. 4A). None of the C-terminal truncated constructs exhibited nuclear localization in the presence of LMB. These results suggest that an NLS is located within the C-terminal half of AZ1, partially overlapping NES2. However, we were unable to further characterize this NLS by mutational analysis, partly because of aggregation of fusion proteins. In addition, no sequences within this region showed any homology with previously known NLS sequences (31, 32). Nevertheless, some nuclear proteins are known to enter the nucleus by binding to importin α or β via an atypical non-basic sequence (33, 34). Alternatively, binding to another nuclear protein may target a protein to the nucleus (35). It is possible that AZ1 enters the nucleus via one of these mechanisms.

Putative Function of AZ1 in the Nucleus—Gritli-Linde et al. (16) reported that antizyme protein is present in the nucleus during mouse development. They also discovered that the proteasome inhibitor MG132 led to the nuclear accumulation of
ODC and suggested that AZ1 is translocated into the nucleus and involved in the degradation of ODC. Our results support the nuclear translocation of AZ1 and provide evidence that AZ1 not only enters the nucleus but also is exported from the nucleus. Consequently, AZ1 may bind to ODC in the nucleus and escort it during cytoplasmic translocation, possibly followed by targeting ODC for the cytoplasmic 26 S proteasomes. Wang et al. (17, 18) reported that AZ forms a complex with Smad1-3 and is translocated into the nucleus. AZ1 may regulate other molecules such as signal transducers or cell cycle regulatory proteins in the nucleus. Because NES1 seems to exert stronger activity than NES2, NES-facilitated nuclear egress is most probably related to functions of the full-length AZ1 (residues 1–227), which is a minor translation product from AZ1 mRNA compared with the product from the second AUG (residues 33–227) (15). Therefore, more active nucleocytoplasmic shuttling, either with or without ODC, might be a specific role of the full-length AZ1.

Most mammalian cells express another family member, AZ2. AZ2 is evolutionarily more conserved than AZ1 (36), but its role is unknown. AZ2 does not have any regions corresponding to the N terminus of AZ1, which includes NES1. However, we found that AZ2 also shuttles between the nucleus and cytoplasm, thus opening the possibility to clarify its novel function.

It is noteworthy that an antizyme fraction was also found in the mitochondrial preparation and that a putative mitochondrion targeting signal (MTS) at the N terminus, which partially overlaps with NES1, is implicated in the mitochondrial localization (15, 37). In our experiments, possible interplay between MTS and NES1 is not addressed, but attaching reporter proteins at the N terminus should inactivate the MTS. Both NES1 and the putative MTS should be present in the short, non-frameshifting translation product between the first AUG and the frameshift site (residues 1–68). This product must be routinely synthesized in the cells irrespective of the cellular polyamine concentration (11). It is currently unknown whether it has any physiological function. The short product, however, would enter the nucleus by diffusion, and if bound to other nuclear molecules such as signal transducers or cell cycle proteins, it would effectively bring them to the cytoplasm as a result of its NES1 activity. Our results suggest that the nucleocytoplasmic shuttling of AZ1 and particularly the nuclear export is a potential regulatory point.

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