REVIEW ARTICLE

Rapid and regulated degradation of ornithine decarboxylase

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INTRODUCTION

Ornithine decarboxylase (ODC; EC 4.1.1.17), the first and key enzyme in polyamine biosynthesis (Figure 1), turns over extremely rapidly and is one of the most highly regulated enzymes known [1–3]. It is induced by many kinds of growth stimuli, and its suppression by specific inhibitors or mutation inhibits cellular growth and transformation [4,5]. A recent study showed that overexpression of ODC caused cellular transformation, suggesting that ODC should be regarded as a proto-oncogene product [6]. ODC is also induced by other types of stimuli, such as hypotonic treatment or differentiation-inducing stimuli, which suggests multiple functions for polyamines [7,8]. On the other hand, ODC is under negative feedback regulation by polyamines for the protection of cells from the adverse effects of excess polyamines [9,10].

The only possible allosteric regulator of ODC known is GTP, which activates ODC of *Escherichia coli*, some tumour cells [11,12] and rat brain [13]. Changes in ODC activity are in general associated with parallel changes in the amount present, except at very low enzyme levels where a significant fraction of ODC may be present as an inactive complex with antizyme [14,15]. The rapid and dramatic regulation of ODC depends on its rapid turnover rate; its half-life usually ranges from several minutes to one hour, which is one of the shortest half-lives of all known

enzymes. Furthermore, ODC degradation is itself regulated, and is accelerated by increases in the levels of cellular polyamines. Recent studies at the molecular level demonstrated that ODC is regulated at four different levels of gene expression, namely gene transcription [16,17], mRNA degradation [17–19], mRNA translation [20,20a,21] and enzyme degradation [3]. In addition, ODC is specifically inhibited by antizyme, a unique regulatory protein of 26.5 kDa. Induced by polyamines, antizyme reversibly binds to ODC with a high affinity (for reviews see [22,23]). Recently, antizyme binding was shown not only to inhibit ODC activity but also to render the enzyme susceptible to proteolysis [24–28]. Studies in cell-free ODC degradation systems revealed that ODC is specifically degraded in an ATP- and antizyme-dependent but ubiquitin (Ub)-independent manner by the cytosolic 26 S proteasome, a multicatalytic protease complex which is widely considered to be specific for Ub-conjugated proteins [26,29].

This review deals mainly with regulation of ODC degradation in higher animals. For wider topics on regulation of ODC and polyamine metabolism, readers are referred to previous reviews [2–5,9,30–32].

HISTORICAL BACKGROUND

Rapid turnover of ODC

The rapid turnover of mammalian ODC was first reported in 1969 by Russell and Snyder [33], who observed that ODC activity in regenerating rat liver decreased upon administration of cycloheximide (an inhibitor of protein synthesis), with a half-life as short as 11 min. This rapid turnover rate is essential for rapid and dramatic changes in the enzyme level in response to stimuli that affect the rate of enzyme synthesis [34]. Furthermore, the turnover rate of ODC is itself subject to marked changes depending on cellular conditions, indicating that its degradation is also regulated. Thus, with various ODC-inducing stimuli, ODC in cultured cells is in general stabilized during the ascending phase of its induction and destabilized during the descending phase [35,36]. These changes in the turnover rate should act, by stimulating both its ascending and descending rates, not only to amplify its induction, but also to make its pattern more pulse-like.

Stabilization of ODC is involved in various types of ODC induction, such as that in the kidney of androgen-treated female mice [37–39] and that in cells treated with amino acids such as asparagine or glutamine [40–42], hypotonic medium [41,43–45], or inhibitors of polyamine synthesis [46–50]. Conversely, ODC is rapidly destabilized by removal of stabilizing amino acids [40], reversal to isotonicity [40,45], or addition of polyamines [51–53]. The destabilization of ODC by polyamines is especially important, since it plays a principal role in the regulation of ODC degradation and its analyses have contributed greatly to elucidation of the mechanism regulating ODC degradation.

Figure 1 Polyamine metabolism in higher animals

Key to enzymes: 1, ornithine decarboxylase (ODC); 2, S-adenosylmethionine decarboxylase (SAMDC); 3, spermidine synthase; 4, spermine synthase; 5, spermidine/spermine N¹-acetyltransferase (SSAT); 6, polyamine oxidase. Abbreviation: MTA, 5'-methylthioadenosine.

Destabilization of ODC by polyamines

ODC in higher animals is subject to negative feedback regulation by polyamines. Thus exogenously added polyamines not only suppress ODC induction in response to various stimuli, but also elicit a rapid decay of preinduced ODC activity [51–53]. Among the three natural polyamines, including the diamine putrescine, spermidine is the most effective and putrescine is the least effective [51,53,54]. The decay of ODC activity induced by sufficiently high doses of polyamines shows a characteristic time course: after an initial lag period of less than 1 h, the enzyme activity decreases at an accelerating rate, which becomes larger than the enzyme decay rate in the presence of cycloheximide [50,55–59]. These findings strongly suggested that ODC degradation is accelerated by polyamines, and this was confirmed by following the decay of prelabelled ODC protein with specific antibodies [58,60]. This fact explains the above-mentioned ODC-stabilizing effect of polyamine synthesis inhibitors, which should deplete cells of polyamines. Importantly, polyamine-induced ODC destabilization was shown to be inhibited by cycloheximide [53,55–57,60,61] but not by actinomycin D [51,57,61], indicating that the effects of polyamines need protein synthesis but not RNA synthesis. In other words, a protein that is induced by polyamines at a post-transcriptional level plays an essential role in destabilizing ODC. This protein also appears to be short-lived, since the ODC-suppressing effect of polyamines injected intraperitoneally lasts for only a few hours in rat liver [62,63].

In addition to the above-mentioned ODC-destabilizing action, polyamines have been thought to exert two more actions that are related to their ODC-suppressing effect, namely suppression of ODC synthesis and induction of antizyme. The former topic will be dealt with later and the latter topic is discussed in the following sections.

Discovery, characterization and role of antizyme

Antizyme was discovered by Canellakis and co-workers, in rat liver and several cultured cells, to be a protein which is induced by polyamines and binds to ODC, inhibiting its activity non-competitively in a stoichiometric manner [64,65]. It is a protein of 26 500 Da, as determined by gel-filtration analysis, and binds to ODC reversibly. The inactive ODC–antizyme complex can be dissociated at high ionic strength and active ODC and antizyme can be separated from each other by gel filtration. Antizyme is also a short-lived protein with a half-life comparable with that of ODC. The induction of antizyme by exogenously added polyamines is inhibited by cycloheximide, but not by actinomycin D.

Antizyme is widely distributed in mammalian and avian tissues and cells (for a review see [23]) as well as in frog liver [66,67]. It was purified over 600 000-fold, to apparent homogeneity, from rat liver [68]. The equilibrium constant of the reaction between antizyme and ODC is as high as $1.4 \times 10^{11} \text{ M}^{-1}$ [68].

As for the metabolic fate of the inactive ODC–antizyme complex, Canellakis and co-workers originally suggested two possibilities: formation of the complex could be a means leading to its more rapid degradation, or it could be a potential store of available ODC or antizyme for cellular requirements [65]. Considerable scepticism prevailed, however, among researchers in this field on the physiological role of antizyme. This was presumably due, at least in part, to the lack of a simple and reliable assay method for the amount of inactive ODC–antizyme complex. Two methods for its assay were developed [50], in which active ODC is released from the complex by excess amounts of either antizyme inhibitor or ODC irreversibly inactivated by treatment with α -difluoromethylornithine

(DFMO). The antizyme inhibitor is a protein originally found in rat liver [69] and binds to antizyme with higher affinity than that of ODC. Analyses of the cellular levels of the ODC–antizyme complex in rat liver and hepatoma tissue culture (HTC) cells revealed that although the amount of the complex increased temporarily on polyamine treatment, the degree of complex formation was far too small to account for the accelerated decrease in ODC activity [50,61,69]. In the absence of exogenously added polyamines, HTC cells contain much lower levels of ODC–antizyme complex than the level of ODC activity; and during the process of ODC induction on change of medium, the amount of the complex increases after the ODC activity reaches a peak, reaching a peak during the descending phase of ODC activity [50]. A similar observation was made in isoprenaline (isoproterenol)-stimulated rat heart [70]. A good correlation exists between the antizyme/ODC ratio and the ODC degradation rate in HTC cells [71]. A similar correlation was observed in the kidneys of male and female mice [39]. On the other hand, the ODC–antizyme complex accumulated markedly in HMO_A cells (ODC-stabilized variant cells derived from HTC cells), suggesting a close relationship between antizyme and ODC degradation [50].

Based on the above observations and the fact that both ODC destabilization and antizyme formation are induced by polyamines, which requires synthesis of a short-lived protein but not RNA, a working hypothesis was presented that antizyme accelerates ODC degradation in a recycling manner [50,71]. If antizyme is essential for ODC degradation, a large proportion, if not all, of the antizyme molecules should act in a recycling manner since the cellular antizyme/ODC ratio is usually much less than unity; and nevertheless ODC is degraded rapidly in the presence of cycloheximide with first-order kinetics.

RECENT PROGRESS

Molecular cloning of antizyme

As the first step in studies on antizyme at the molecular level, several monoclonal antibodies against rat liver antizyme were obtained [72]. A partial antizyme cDNA (Z1) was cloned using the monoclonal antibodies as probes [73]. The authenticity of this clone was verified by expression of active (ODC-inhibitory) antizyme from the cDNA in bacterial expression systems. The Z1 antizyme cDNA and its variants have been used in various laboratories to explore the roles of antizyme [24,27,74–77]. Using the Z1 antizyme cDNA as a probe, full-length cDNA [78] and genomic [79] clones were isolated.

The rat antizyme gene consists of five exons and four introns and is driven by a potent promoter [79]. Northern-blot analyses show that antizyme mRNA is amply expressed constitutively in various tissues of rats, with a half-life in liver of as long as 12 h, and that the level of antizyme mRNA is not further elevated by administration of polyamines. These findings indicate that polyamines induce antizyme by stimulating the translation of its mRNA [73], in accordance with earlier observations that antizyme induction is not inhibited by actinomycin D.

Polyamines induce antizyme by evoking translational frameshifting

The entire nucleotide sequence of antizyme mRNA determined by the cDNA [78] and gene [79] analyses showed that antizyme is encoded in two open reading frames (ORFs) (Figure 2a). The initiating frame (ORF1) is short, encoding only 68 amino acids. The second, and main, frame (ORF2) is in the +1 frame with respect to ORF1, and this frame lacks possible initiator codons.

Figure 2 Frameshift in translation of antizyme mRNA

(a) Diagram of rat liver antizyme mRNA. Red-tinted boxes indicate two major open reading frames (ORF1, ORF2), and black bars indicate untranslated regions. The positions of relevant initiation (AUG) and termination (UAG, UGA) codons are indicated. The 5'-end of the partial cDNA, Z1, is indicated. An means poly(A). The circled region contains the frameshift site and two downstream stimulators (see text), which are shown below in detail. Note that an artificial initiator or a 5'-fusion gene is placed 5' to Z1 in-phase of ORF2 in expression studies using Z1, and therefore their products contain unnatural peptide encoded in the part of ORF2 overlapping to ORF1. (b) A potential pseudoknot structure. The terminator codon of ORF1 is enclosed by an octagon. (c) +1 Frameshift at the end of ORF1. A transframe peptide derived from a fusion construct expressed in reticulocyte lysate was sequenced using radio-sequencing technique [78]. The positions of cysteine, serine and proline have been determined.

Cell-free translation in rabbit reticulocyte lysate revealed that the change of the reading frame is accomplished by translational frameshifting, which is elicited by addition of polyamines [78,80]. This was confirmed recently by Rom and Kahana [81,82]. The site and direction of the frameshifting were determined by amino acid radio-sequencing of a transframe product [78]. The sequence at the 3' end of ORF1, UCC-UGA-U, which includes a termination codon (underlined), is decoded as Ser-Asp in the frameshift product (Figure 2c), and thus the frameshifting is +1. Mutational analysis across the shift site suggested that repairing of peptidyl-tRNA in the new frame is not an absolute requirement for the shift [78].

Known cases of frameshifting are usually accompanied by one or two elements (stimulators) on mRNA which enhance the frameshift efficiency [83]. Two stimulators have been identified in antizyme frameshifting (Figure 2b). The terminator codon of ORF1 is essential. A pseudoknot structure (Figure 2b) which is located 3' to the terminator stimulates the frameshifting by 2–3-fold. The region consisting of the shift site and the downstream pseudoknot directs polyamine-dependent frameshifting in a foreign context, indicating that all of the essential sequence for the frameshifting is located within this region. Spermidine, spermine and putrescine show the stimulatory effect. The shift

efficiency is 19% at the optimal concentration of spermidine, as determined on a fusion construct [78].

Since antizyme indirectly affects the cellular polyamine levels as discussed below, the polyamine-dependent frameshifting is an integral part of an autoregulatory circuit. It serves as a sensor for modulating the cellular concentration of polyamines. However, the mechanism by which polyamines stimulate frameshifting remains to be clarified. Polyamines do not appear to act by modulating the stimulator activity of the downstream pseudoknot or the terminator codon, since frameshifting of mutant constructs lacking one or both of these stimulators still respond to polyamines [78].

There are several exciting features in antizyme frameshifting. It is the first programmed frameshifting in the decoding of an eukaryotic cellular gene although the number of examples of programmed frameshifting is increasing, particularly in the decoding of compact genomes such as retrotransposons and viruses [84]. The mechanism involved is distinct from that of the frameshifting used in decoding various virus genes, as well as all other known cases. Frameshifting is a novel mechanism for modulation of gene expression in eukaryotic cells; however, in bacteria, the synthesis of polypeptide chain release factor 2 is known to be controlled through autoregulatory frameshifting [85].

Antizyme destabilizes ODC

Definite proof of the essential role of antizyme in ODC degradation was obtained by examining the effect of expressing transfected antizyme cDNA [24]. HTC cells were transfected with an expression vector, pMAMneoZ1, in which the N-terminal-truncated antizyme cDNA Z1, with a linker serving as an initiator codon, was constructed downstream of a glucocorticoid-inducible promoter. In cells of a stable transfectant clone thus obtained (HZ7 cells), a synthetic glucocorticoid, dexamethasone, causes significant induction of active antizyme in the absence of exogenous polyamines and elicits an accelerating decay of ODC activity, as well as ODC protein. This decay becomes more rapid than ODC decay in the presence of cycloheximide. The ODC destabilization is inhibited by cycloheximide. All of these results, which are not obtained in control transfectant cells, are essentially the same as those obtained after addition of polyamines, except for a somewhat longer lag period before the rapid ODC decay.

This lag period can be well explained by the requirement of both transcription and translation for the expression of transfected antizyme cDNA, whereas only translation is required for antizyme induction by polyamines. Thus these results clearly indicate that antizyme mediates the effect of polyamines in destabilization of ODC.

The role of antizyme was further confirmed by the following two lines of evidence. First, mouse–trypanosome chimera ODCs, which cannot bind antizyme, are no longer subject to polyamine-induced degradation [27,28]. Secondly, ODC degradation in cell-free systems is markedly stimulated by antizyme [25,26,28].

Recently, antizyme was also found to play an important role in the regulation of polyamine transport. Polyamine uptake is known to be under negative feedback regulation, in which a labile protein is induced by polyamines and reversibly inactivates the polyamine transporter [86,87]. Utilizing the glucocorticoid-inducible Z1 antizyme-transfected cell line HZ7, Mitchell and coworkers clearly demonstrated that dexamethasone treatment induces antizyme activity and elicits a decrease of spermidine transport activity [76]. Essentially the same result was obtained by Suzuki et al. [77]. It is interesting in this connection to note that

Figure 3 Antizyme-dependent ODC degradation in cell extracts

Extracts of DF3 cells (ODC-overproducing CHO cells) containing metabolically labelled ODC were incubated with various amounts of affinity-purified recombinant antizyme and an ATP-generating system. At 30 min and 60 min, labelled ODC remaining in the mixture was immunoprecipitated and analysed by SDS/PAGE. After fluorography, the radioactivity of the ODC band was measured. (a) Time courses of ODC degradation. (b) Dose-response curve for antizyme. The half-lives were determined from the decreases in labelled ODC during the initial 30 min incubation. (Reproduced from [25] with permission).

the presence of membrane-bound antizyme has been repeatedly observed [56,88,89] and that the membrane-bound antizyme in mouse kidney is also inducible by polyamines [39]. Thus antizyme is a bifunctional regulatory protein that plays a pivotal role in the negative feedback system to prevent excess accumulation of cellular polyamines. Furthermore, antizyme may play another role, namely apparent suppression of ODC synthesis at a post-translational level. This will be discussed later.

In vitro ODC degradation systems

Analysis in a cell-free system is obviously important for elucidation of the molecular mechanism of any cellular function. The selective proteolytic machinery, however, appears to be more fragile than the protein synthesis system, and establishment of a cell-free ODC degradation system took a long time. Although several *in vitro* ODC inactivation systems have been reported, these are probably not related to physiological ODC degradation in cells [90–92]. Several years ago, Kahana and co-workers developed an *in vitro* ODC degradation system [93]. They showed that ^{35}S -labelled ODC synthesized *in vitro* in their reticulocyte lysate is degraded in that reticulocyte lysate system in the presence of ATP. Ub is not needed as ODC degradation in their system was not affected by removal of Ub, Ub-activating enzyme E1, or all of the three enzymes, E1, E2 and E3, involved in protein ubiquitination. These features are in accordance with features of ODC degradation in intact cells [94]. Their *in vitro* system, however, is not active with purified exogenous ODC isolated from other cells, leaving some doubt as to the physiological role of the system. This doubt was resolved 4 years later as described below.

It was recently demonstrated [25] that in cell-free extracts of ODC-overproducing Chinese hamster ovary (CHO) cells (DF3) [95] and HTC cells both endogenous and exogenous ODC are degraded well in the presence of ATP and antizyme. Antizyme acts in a dose-dependent manner and its maximum effect is obtained when the molar ratio of antizyme to ODC monomer approaches unity (Figure 3), indicating that antizyme acts by

binding with ODC in a one-to-one ratio. The fact that a relatively small amount of antizyme can accelerate the degradation of much larger amounts of ODC indicates that, as *in vivo*, antizyme acts in a recycling manner. In another study with ODC-overproducing mouse cells (EXOD-1 cells), extracts of cells that had been precultured with spermidine to accumulate antizyme exhibited substantial activity to degrade ODC in the absence of exogenous antizyme, and removal of antizyme by immunoprecipitation resulted in almost complete loss of ODC-degrading activity [96]. These results support not only the essential role of antizyme in ODC degradation, but also the physiological nature of the *in vitro* ODC degradation system.

The possible role of antizyme was examined in the reticulocyte lysate ODC degradation system developed by Kahana and co-workers [97]. Efficient degradation of exogenous ODC was observed in a reticulocyte lysate in the presence of ATP and in the absence of exogenous antizyme, when a small amount of ^{35}S metabolically labelled and affinity-purified ODC was used as an exogenous substrate. Degradations of both endogenous and exogenous ODCs were markedly suppressed by antizyme inhibitor. These results indicated that ODC degradation in reticulocyte lysates is also dependent on antizyme and therefore reflects physiological ODC degradation in reticulocytes. Thus, the amount of endogenous antizyme in reticulocyte lysates is so minute that although it is enough for ODC synthesized *in vitro*, it is not sufficient for relatively large amounts of exogenous ODC.

ODC is degraded by the 26 S proteasome, without ubiquitination

Establishment of a cell-free system for ODC degradation made it possible to identify the protease responsible. Three major intracellular proteases are known, namely lysosomal cathepsins, cytosolic calpains and multicatalytic proteinases (proteasomes) [98]. First, the effects of various inhibitors on the *in vitro* ODC degradation were examined [26]. High levels of EGTA or E64, which both inhibit calpain, did not inhibit ODC degradation. Peptide inhibitors of lysosomal proteases such as leupeptin and

Figure 4 Antizyme-dependent ODC degradation pathway catalysed by 26 S proteasome without ubiquitination

Also suppressing polyamine transport, antizyme plays pivotal roles in preventing over-accumulation of polyamines. Abbreviations: AZ, antizyme; Ub, ubiquitin; Put, putrescine; Spd, spermidine; Spm, spermine.

chymostatin, or inhibitors of serine proteases, such as soybean trypsin inhibitor, were also not inhibitory, indicating that the protease responsible for ODC degradation was not calpain or any lysosomal protease. On the other hand, *in vitro* degradation of ODC was strongly inhibited by haemin, which blocks ATP-dependent degradation of ubiquitinated proteins, resulting in accumulation of high-molecular-mass Ub-protein conjugates [99]. No such high-molecular-mass bands were detected in the fluorogram upon SDS/PAGE analysis of the reaction mixture after ODC degradation with or without haemin. These results suggest that ODC may be degraded by proteasomes. Ubiquitination, however, seems not to be involved, in accordance with previous *in vivo* [94] and *in vitro* [93] observations. Non-hydrolysable analogues of ATP were unable to replace ATP, indicating that ATP was needed as an energy source.

The above results prompted us to examine the possible participation of proteasomes, major cytosolic protease complexes that catalyse energy-dependent proteolysis. Removal of proteasomes from HTC cell extracts by immunoprecipitation with anti-proteasome antibody decreased both the ODC-degrading activity and proteasome activity measured with a fluorogenic peptide substrate. Both showed identical dose-response relationships, and a large dose of anti-proteasome antibody caused almost complete loss of both activities. This indicated that proteasomes are essentially involved in ODC degradation. Proteasomes are known to exist in two types, namely 20 S proteasomes (700 kDa), consisting of multiple subunits of 21–31 kDa, and 26 S proteasomes (2000 kDa), which are made up of a 20 S proteasome core and many associated regulatory subunits of 35–110 kDa. The 20 S proteasome shows multicatalytic protease activities with or without ATP, whereas the 26 S proteasome catalyses ATP-dependent degradation of

Figure 5 Structures of mouse and trypanosome ODCs

polyubiquitinated proteins [100–105]. Upon fractionation of highly purified 26 S proteasome by glycerol density-gradient centrifugation, the profile of ODC-degrading activity exactly coincided with the profiles of 26 S proteasome activity measured against the fluorogenic peptide substrate or ^{125}I -lysozyme-Ub conjugate. No ODC-degrading activity was observed in the 20 S proteasome fraction. Degradation of ODC by the purified 26 S proteasome, which does not contain Ub or Ub-conjugating enzymes, requires ATP hydrolysis and is antizyme-dependent. These results clearly indicate that the 26 S proteasome is responsible for ODC degradation [26]. This is the first demonstration that a natural enzyme is degraded *in vitro* by a specific proteinase in an ATP-dependent manner. This is also the first established case of degradation of a non-ubiquitinated protein by the 26 S proteasome, which has been widely regarded as being specific to ubiquitinated proteins (Figure 4). Possibly, some other short-lived enzymes are also degraded by the 26 S proteasome without ubiquitination. Kahana and co-workers confirmed that ODC is degraded by the proteasome [29]. They also demonstrated that both yeast and mouse ODCs are rapidly degraded in wild-type yeast cells, but are dramatically stabilized in yeast cells harbouring a mutant proteasome (yscE pronase) defective in chymotryptic activity [106]. These results provide genetic evidence for the involvement of the proteasome in the degradation of ODC *in vivo*.

Structural elements of ODC and antizyme responsible for ODC degradation

The importance of the C-terminal region for the rapid degradation of ODC was brought to light mainly by the studies of Coffino and co-workers on chimeras and deletion mutants of mouse and trypanosome ODCs. In marked contrast with mammalian counterparts, trypanosomal ODC is quite stable in the parasite cells [107]. This may contribute to the effectiveness of DFMO in suppressing ODC activity and in effecting the cure of African trypanosomiasis in mice [108] and humans [109]. Sequence analysis of the ODC gene cloned from *Trypanosoma brucei* revealed that the parasitic ODC has 61.5% sequence similarity with the corresponding sequence of the mouse enzyme. As shown in Figure 5, major discrepancies are the addition of a 20-amino-acid N-terminal peptide to, and the deletion of a 36-amino-acid C-terminal peptide from, the trypanosomal ODC [107]. The stability of trypanosomal ODC is a property of the ODC protein itself rather than of the cellular environment, since trypanosomal ODC expressed in mammalian cells is also stable [110]. The C-terminus of mouse ODC has been assumed to be one of the structural elements associated with rapid turnover of the enzyme since its sequence is conserved in mammalian ODCs but is dispensable for catalytic activity, suggesting its important

non-enzymic function [111]. In addition, it contains one of the two PEST sequences of ODC (amino acid residues 298–333 and 423–449). These regions are rich in proline (P), glutamic acid (E), serine (S) and threonine (T) and were postulated by Rechsteiner and co-workers [112,113] to play an important role in the selective degradation of short-lived proteins (PEST hypothesis). Mouse ODC is stabilized in whole cells or *in vitro* systems by total or partial deletion of the C-terminal region of 36 to 38 amino acids [114–116]. Fusion of the mouse C-terminus of 37 amino acids to either end of dihydrofolate reductase conferred *in vitro* instability to the stable enzyme [117]. Even a single amino acid replacement (Cys-441 to Trp-441) resulted in ODC stabilization in HMO_A cells [118]. These findings indicate that the C-terminal region of ODC acts as a signal for its rapid proteolysis. It should be noted that although the second PEST region is included in the C-terminal region, which is necessary and sufficient for constitutive degradation of ODC, other nearby regions are also effective. Thus truncation of five C-terminal amino acids, which are outside the PEST region, also stabilizes ODC [119]. The other PEST region (amino acid residues 298–333) is totally dispensable and therefore has no role in ODC degradation [119]. Thus, although the PEST sequence appears to be related to rapid proteolysis, it alone is not sufficient as a signal.

Trypanosomal ODC does not interact with rat antizyme *in vitro* [27]. Examination of a series of enzymically active chimeric or deletion mutants of mouse and trypanosome ODCs for their interaction with antizyme *in vivo* and *in vitro* revealed that an internal region of mouse ODC (amino acids 117 to 140) is both necessary and sufficient for antizyme binding and inhibition of ODC activity, but not for polyamine-dependent acceleration of ODC degradation, which needs in addition the C-terminal region [27,28]. Thus the constitutive and polyamine-induced ODC-degrading pathways converge at a process in which the C-terminal region is involved. It is likely that the C-terminal region is recognized and attacked by the 26 S proteasome, whereas antizyme binding induces a conformational change in the ODC molecule resulting in exposure of the C-terminal region to the 26 S proteasome. In fact antizyme was shown to stimulate the binding of anti-(C-terminus) antibody to mouse ODC [28].

In an extension of their survey of the functional domains of ODC involved in ODC–antizyme interactions, Li and Coffino [74] revealed that the C-terminal half of antizyme (amino acids 106–212) alone can bind to ODC and inactivate it but cannot accelerate its degradation, which requires in addition an adjacent region (amino acids 55–105). Essentially the same results were obtained independently by Ichiba et al. [75]. These results suggest that the binding of antizyme elicits a conformational change in ODC, resulting in exposure of a hidden degradation signal to the proteolytic system.

Tentative model of antizyme-stimulated ODC degradation

Mouse ODC is a homodimer of a subunit with 461 amino acid residues [120,121]. Two active sites are located at the interface between the subunits and each active site is made up of amino acid residues from both subunits [122,123]. This is consistent with a previous observation using gel-filtration chromatography that the active ODC dimer is dissociated into inactive monomers by the addition of high concentrations of salts [68,124,125]. The two subunits dissociate and reassociate unusually rapidly, so that randomization is complete within 5 min under physiological conditions [123]. Antizyme binds preferentially to the ODC monomer [126]. The rapid dissociation and reassociation of the ODC subunits should be advantageous for antizyme binding, fixing the enzyme in the inactive monomer form and eliciting its

Figure 6 Tentative model of antizyme (AZ)-dependent degradation of ODC by 26 S proteasome

conformational change to expose its C-terminus, which is susceptible to degradation (Figure 6). Consistent with this model, ODC subunit recognition by the proteolytic machinery occurs only in the *cis* subunit, not in the *trans*. Thus, when degradation of an ODC heterodimer composed of one unstable wild-type subunit and one stable mutant subunit was tested in a reticulocyte lysate, each subunit maintained its own degradation characteristics and was not affected by its partner subunit [127]. Also consistent with this model, ODC mutants in which well-conserved Gly-387 is replaced by any other amino acid except alanine are unable to form a stable dimer and are short-lived [128].

Very recently, ODC, complexed with a recombinant antizyme, was found to be degraded by the 26 S proteasome into various peptides of 5–11 amino acid residues with various C-terminal amino acids. These peptides were derived from regions scattered throughout the ODC sequence, without liberation of free amino acids [129]. This indicates, for the first time with a natural substrate protein, that the 26 S proteasome acts as a multi-functional endoprotease complex. This finding supports the recent proposal that 26 S proteasome is responsible for the processing of antigens into short peptides to be bound to major histocompatibility complex (MHC) class I molecules (for reviews see [130,131]). The recombinant antizyme was not degraded at all during ODC degradation, indicating that degradation of

antizyme is not necessary for ODC degradation. This does not mean, however, that natural antizyme is not degraded at all, since the recombinant antizyme used was a stable fusion protein with maltose-binding protein. Although the exact mechanism is unknown, a part of antizyme is likely to be degraded during recycling (Figure 4), since ODC degradation in the presence of cycloheximide usually proceeds with first-order kinetics (fixed rate), which means that the antizyme/ODC ratio is kept almost the same during degradation of ODC [25].

Several advantages of the antizyme-mediated ODC decay-regulating system are conceivable. First, this system offers rapid, effective and highly selective negative feedback regulation of cellular polyamine levels, since both ODC degradation and polyamine transport are regulated in concert by the specific actions of antizyme, which is short-lived and rapidly induced by polyamines at the translational level. Secondly, while most of the cellular spermidine and spermine are bound to RNA and DNA [10,132], this regulatory system, being based on polyamine-induced frameshifting, may well reflect the free/bound state of cellular polyamines and therefore be an excellent system of negative feedback regulation. Thirdly, ODC induction is both amplified and made more pulse-like, as the antizyme/ODC ratio, which determines the rate of ODC degradation, falls during the initial phase of ODC induction due to increases in ODC level and then rises due to an increase in the antizyme level. This would allow rapid and transient increases in cellular polyamine levels without causing excess accumulation of polyamines that are cytotoxic.

Comparative biochemistry

The unique negative feedback regulation of ODC mediated by antizyme has mainly been studied in tissues and cells of rats and mice. Comparative studies of the ODC/antizyme system in a wide variety of organisms may cast light on the question of how such a unique system has been developed during biological evolution, in particular the extremely rapid turnover of ODC, the role of antizyme, and the frameshifting-based mechanism of antizyme induction. Available evidence suggests that the ODC/antizyme system is widely distributed in mammals (for a review see [23]), birds [133] and amphibians [66,67]. Northern-blot analysis showed the presence of antizyme mRNA of identical size and which was able to cross-hybridize with rat antizyme cDNA in human, chicken and frog livers [73]. Frog antizyme cDNA shows 63% sequence similarity with rat antizyme cDNA (T. Ichiba, S. Matsufuji and S. Hayashi, unpublished work).

In lower eukaryotes such as yeasts [134,135], *Neurospora* [136,137] and protozoans [138,139], ODC is moderately short-lived (half-life about 1 h) and antizyme has not been detected, although various types of negative feedback regulation appear to operate. These include protein synthesis-dependent ODC destabilization observed in *Neurospora crassa* [136,140]. In some parasitic protozoans, such as *Trypanosoma brucei* [107] and *Leishmania donovani* [141], ODC is exceptionally stable and not regulated, possibly owing to the homeostatic environment. A quite different mechanism works in *Physarum polycephalum*. Thus, polyamines induce reversible conversion of active A form into less active B form, which is catalysed by ODC-modifying protein [142,143].

In prokaryotes, three antizymes of *E. coli* have been found [144] and later identified as two ribosomal proteins, S20/L26 and L34 [145,146] and a transcriptional regulator [147]. Although the physiological roles of *E. coli* antizymes remain to be clarified, these are not related to ODC degradation, since *E. coli* ODC is

quite stable [147]. In plants, some antizyme-like ODC-inhibitory proteins [148] and short-lived ODC [149] have been reported, but the relationships of these proteins with ODC degradation are unknown. Thus, it is important to extend the survey to organisms in between vertebrates and protozoans.

Unsolved problems

There have been reports of antizymes of various sizes in addition to the 26.5 kDa form that corresponds to the size of the known mRNA. Thus, antizymes of 50 kDa have been reported to be present in rat thymus [150] and mammary gland [151], and even antizyme of 90 kDa was observed in lymphoma cells [152]. Northern-blot analysis indicated that thymus extract contained a single hybridizable antizyme mRNA of a size identical with that of rat liver antizyme mRNA [73]. The 50 kDa antizyme may be antizyme dimer formed from the monomer by an unknown mechanism.

As described before, ODC is stabilized severalfold when cells are exposed to hypotonic medium [44,45] or shifted from salt-glucose medium to a medium containing amino acids that belong to the A transport system, such as asparagine and glutamine [40–42]. Conversely, rapid destabilization occurs upon removal of the stabilizing factors. Further studies are required to elucidate the exact mechanisms underlying these changes in ODC stability.

The physiological role of antizyme inhibitor remains to be clarified. It was discovered in rat liver as a protein that binds to antizyme with a higher affinity than that of ODC [69]. Kitani and Fujisawa purified it 17000000-fold to apparent homogeneity from rat liver [153]. Like ODC, it is a homodimer of a 50 kDa subunit and cross-reacts with anti-ODC antibody very weakly [154]. Its level in rat liver remained constant after administration of putrescine in marked contrast with the ODC level, which decreased rapidly. This suggests that antizyme inhibitor is formed independently of ODC. Its rapid turnover (half-life less than 20 min) and the pattern of diet-induced change in its level in rat liver, as compared with changes in levels of ODC and ODC-antizyme complex, suggest that it is another regulatory protein that stabilizes ODC by trapping antizyme [154]. Similar observations were made in the hearts of isoprenaline-stimulated rats [70,154]. Cloning of its cDNA is essential to clarify its structure and function.

Translational repression of ODC synthesis, together with acceleration of its degradation, have been widely considered to be responsible for ODC repression by polyamines [20,21]. Coffino and co-workers challenged this general concept by postulating that the suppression of [³⁵S]methionine incorporation into ODC by polyamines is due to stimulation of rapid degradation of newly synthesized ODC [155]. Thus, differences between polyamine-starved and polyamine-replete cells in the incorporation of radioactive methionine into ODC diminished progressively with a decrease in pulse-label time, reaching a minimum with a pulse time of 4 min. In similar types of studies, Persson and co-workers did not find that the difference was abolished [156]. The reason for the discrepancy is not known at present. In accordance with the postulation of Coffino and co-workers, dexamethasone-induced accumulation of antizyme in HZ7 cells suppressed [³⁵S]methionine incorporation into ODC (10 min pulse-labelling) by more than 50%, and this was not due to direct suppression of ODC synthesis at the translational level, since *in vitro* translation of ODC mRNA was not inhibited by antizyme [157]. It should be pointed out that ODC-stabilizing conditions, such as polyamine depletion, addition of asparagine, and hypotonic treatment, are also conditions under which

incorporation of radioactive methionine into ODC is apparently stimulated at the translational level. It should also be noted that a single treatment of cells with putrescine affected both [³⁵S]methionine incorporation into ODC and degradation of ODC with exactly the same time course [58]. Since newly synthesized ODC is a monomer that should bind with antizyme much more easily than preformed ODC, which is mostly a dimer, it is reasonable to suggest that newly synthesized ODC turns over much more rapidly than preformed ODC. In fact, the rate of degradation of ODC complexed with antizyme is extremely rapid, its half-life being less than 5 min [157]. Accordingly, the concept of Coffino and co-workers seems to fit well with the tentative model of ODC degradation (Figure 6). Persson and co-workers recently reported that COS cells overexpressing a transfected ODC transcript devoid of the 5'-untranslated region did not show any polyamine-induced repression under conditions where the mock-transfected cells did [21]. It should be noted, however, that the ODC level in the ODC-transfected cells was 250 times as high as that in the mock-transfected cells and therefore a much higher concentration of polyamine was probably needed to produce enough antizyme for acceleration of ODC decay in these cells. In any case, regulation of ODC degradation is more important than was thought previously.

CONCLUDING REMARKS AND PERSPECTIVES

ODC, which turns over in a regulated manner with variable half-life ranging from minutes to more than 10 h, is probably one of the most attractive targets for researchers interested in selective proteolysis and/or metabolic regulation. Much progress has been made in the last five years about the molecular mechanism underlying the polyamine-regulated ODC degradation in mammalian cells, thanks to gene technology. As reviewed in this article, the mechanism is unique, with many novel features. First, the overall mechanism involving antizyme is a novel type of negative feedback regulation. Secondly, polyamines induce antizyme by evoking translational frameshifting, an extremely unusual event. Finally, ODC complexed with antizyme is degraded by the 26 S proteasome without ubiquitination, indicating a novel function for the protease complex. These novel characteristics do not necessarily mean that they will continue to be unique to ODC. It is expected that similar cases will be found in the future for each of the unique features of ODC/antizyme system.

Antizyme turned out to be a bifunctional regulatory protein which not only destabilizes ODC but also inactivates the polyamine uptake system, and therefore effectively prevents excess accumulation of polyamines. Future studies might reveal that antizyme mediates some physiological function(s) of polyamines related to growth and differentiation, since growth retardation of HZ7 cells caused by forced expression of a transfected antizyme gene could not be totally recovered by supplementation of spermidine [157]. The physiological role of antizyme should be examined using mutant cells that either lack or overproduce antizyme. Knockout mice should also be useful. Chromosomal mapping of the human antizyme gene might lead to specification of some human disease(s) due to a hereditary disorder of antizyme. X-ray crystallography should be a good method for studying the interaction of ODC, antizyme and 26 S proteasome.

Characteristics of antizyme frameshifting should be verified using an *in vivo* system such as transfected cultured cells. The precise mechanism of the frameshifting, especially that of the polyamine effect, should also be studied. Yeast systems may enable a genetic approach to be used, which is especially useful

for studying possible *trans*-acting factors involved (such as tRNAs, elongation factors and ribosomal components). These studies may open up a new perspective into both the mechanism of elongation and the physiological roles of polyamines.

As for 26 S proteasome, important problems that remain to be solved include specification of the proteasome subunit that binds to ODC complexed with antizyme, and the mechanism of ATP requirement. Intracellular selective proteolysis is known to be ATP-dependent [100]. In the case of ODC degradation, selection is accomplished by antizyme binding without using energy. It is intriguing to reveal why and how ATP hydrolysis is needed for ODC degradation, which is basically an exergonic reaction.

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REFERENCES

- 1 Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749–790
- 2 Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- 3 Hayashi, S. (1989) in *Ornithine Decarboxylase: Biology, Enzymology, and Molecular Genetics* (Hayashi, S., ed.), pp. 35–45, Pergamon Press, New York
- 4 Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- 5 McCann, P. P. and Pegg, A. E. (1992) *Pharmacol. Ther.* **54**, 195–215
- 6 Auvinen, M., Paasinen, A., Andersson, L. C. and Hölttä, E. (1992) *Nature (London)* **360**, 355–358
- 7 Pegg, A. E. and McCann, P. P. (1982) *Am. J. Physiol.* **243**, C212–C221
- 8 Marton, L. J. and Morris, D. R. (1987) in *Inhibition of Polyamine Biosynthesis: Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E. and Sjoerdsma, A., eds.), pp. 79–105, Academic Press, New York
- 9 Morris, D. R. (1991) *J. Cell Biochem.* **46**, 102–105
- 10 Davis, R. H., Morris, D. R. and Coffino, P. (1992) *Microbiol. Rev.* **56**, 280–290
- 11 O'Brien, T. G., Madara, T., Pyle, J. A. and Holmes, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9448–9452
- 12 Hietala, O., Dzubow, L., Dlugosz, A. A., Pyle, J. A., Jenney, F., Gilmore, S. K. and O'Brien, T. G. (1988) *Cancer Res.* **48**, 1252–1257
- 13 Kilpeläinen, P. T. and Hietala, O. A. (1994) *Biochem. J.* **300**, 577–582
- 14 Fujita, K., Matsufuji, S., Murakami, Y. and Hayashi, S. (1984) *Biochem. J.* **218**, 557–562
- 15 Onoue, H., Matsufuji, S., Nishiyama, M., Murakami, Y. and Hayashi, S. (1988) *Biochem. J.* **250**, 797–803
- 16 Greenberg, M. E., Green, L. A. and Ziff, E. B. (1985) *J. Biol. Chem.* **260**, 14101–14110
- 17 Abrahamsen, M. S. and Morris, D. R. (1990) *Mol. Cell. Biol.* **10**, 5525–5528
- 18 Hölttä, E., Sistonen, L. and Alitalo, K. (1988) *J. Biol. Chem.* **263**, 4500–4507
- 19 Chen, Z. P. and Chen, K. Y. (1992) *J. Biol. Chem.* **267**, 6946–6951
- 20 Kameji, T. and Pegg, A. E. (1987) *J. Biol. Chem.* **262**, 2427–2430
- 20a Ito, K., Kashiwagi, K., Watanabe, S., Kameji, T., Hayashi, S. and Igarashi, K. (1990) *J. Biol. Chem.* **265**, 13036–13041
- 21 Lökvist, E., Stjernborg, L. and Persson, L. (1993) *Eur. J. Biochem.* **215**, 753–759
- 22 Canelakakis, E. S. and Hayashi, S. (1989) in *The Physiology of Polyamines* (Bachrach, U. and Heimer, Y. M., eds.), vol. I, pp. 315–330, CRC Press, Boca Raton
- 23 Hayashi, S. and Canelakakis, E. S. (1989) in *Ornithine Decarboxylase: Biology, Enzymology, and Molecular Genetics* (Hayashi, S., ed.), pp. 47–58, Pergamon Press, New York
- 24 Murakami, Y., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1992) *J. Biol. Chem.* **267**, 13138–13141
- 25 Murakami, Y., Tanaka, K., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1992) *Biochem. J.* **283**, 661–664
- 26 Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K. and Ichihara, A. (1992) *Nature (London)* **360**, 597–599
- 27 Li, X. and Coffino, P. (1992) *Mol. Cell. Biol.* **12**, 3356–3362
- 28 Li, X. and Coffino, P. (1993) *Mol. Cell. Biol.* **13**, 2377–2383
- 29 Bercovich, Z. and Kahana, C. (1993) *Eur. J. Biochem.* **213**, 205–210
- 30 Heby, O. and Persson, L. (1990) *Trends Biochem. Soc.* **15**, 153–158
- 31 Davis, R. H. (1990) *J. Cell Biochem.* **44**, 199–205
- 32 Jänne, J., Alhonen, L. and Leinonen, P. (1991) *Ann. Med.* **23**, 241–259
- 33 Russell, D. H. and Snyder, S. H. (1969) *Mol. Pharmacol.* **5**, 253–262
- 34 Berlin, C. M. and Schimke, R. T. (1965) *Mol. Pharmacol.* **1**, 149–156
- 35 Hogan, B. L. M. and Murden, S. (1974) *J. Cell. Physiol.* **83**, 345–352
- 36 Prouty, W. F. (1976) *J. Cell. Physiol.* **89**, 65–76
- 37 Seely, J. E., Pösö, H. and Pegg, A. E. (1982) *J. Biol. Chem.* **257**, 7549–7553
- 38 Isomaa, V. V., Pajunen, A. E. I., Bardin, C. W. and Jänne, O. (1983) *J. Biol. Chem.* **258**, 6735–6740

- 39 Murakami, Y., Marumo, M. and Hayashi, S. (1988) *Biochem. J.* **254**, 367–372
- 40 Chen, K. Y. and Canellakis, E. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3791–3795
- 41 Viceps-Madore, D., Chen, K. Y., Tsou, H.-W. and Canellakis, E. S. (1982) *Biochim. Biophys. Acta* **717**, 305–315
- 42 Kanamoto, R., Boyle, S. M., Oka, T. and Hayashi, S. (1987) *J. Biol. Chem.* **262**, 14801–14805
- 43 Munro, G. F., Miller, R. A., Bell, C. A. and Verderb, E. L. (1975) *Biochim. Biophys. Acta* **411**, 263–281
- 44 Poulin, R. and Pegg, A. E. (1990) *J. Biol. Chem.* **265**, 4025–4032
- 45 Tohyama, Y., Kameji, T. and Hayashi, S. (1991) *Eur. J. Biochem.* **202**, 1327–1331
- 46 Harik, S. I., Hollenberg, M. D. and Snyder, S. H. (1974) *Mol. Pharmacol.* **10**, 41–47
- 47 Inoue, H., Kato, Y., Takigawa, M., Adachi, K. and Takeda, Y. (1975) *J. Biochem. (Tokyo)* **77**, 879–893
- 48 McCann, P. P., Tardif, C., Dechesne, M. C. and Mamont, P. S. (1977) *Biochem. Biophys. Res. Commun.* **76**, 893–899
- 49 Karvonen, E. and Pösö, H. (1984) *Biochim. Biophys. Acta* **791**, 239–243
- 50 Murakami, Y., Fujita, K., Kameji, T. and Hayashi, S. (1985) *Biochem. J.* **225**, 689–697
- 51 Kay, J. E. and Lindsay, V. J. (1973) *Biochem. J.* **132**, 791–796
- 52 Jänne, J. and Hölttä, E. (1974) *Biochem. Biophys. Res. Commun.* **61**, 449–456
- 53 Seely, J. E. and Pegg, A. E. (1983) *Biochem. J.* **216**, 701–707
- 54 Heller, J. S., Chen, K. Y., Kyriakidis, D. A. and Canellakis, E. S. (1978) *J. Cell. Physiol.* **96**, 225–234
- 55 Pösö, H., Guha, S. K. and Jänne, J. (1978) *Biochim. Biophys. Acta* **524**, 466–473
- 56 McCann, P. P., Tardif, C., Hornsperger, J. M. and Bohlen, P. (1979) *J. Cell. Physiol.* **99**, 183–190
- 57 Mitchell, J. L. A., Mahan, D. W., McCann, P. P. and Qasba, P. (1985) *Biochim. Biophys. Acta* **840**, 309–316
- 58 Kanamoto, R., Utsunomiya, K., Kameji, T. and Hayashi, S. (1986) *Eur. J. Biochem.* **154**, 539–5445
- 59 Glass, J. R. and Gerner, E. W. (1987) *J. Cell. Physiol.* **130**, 133–141
- 60 Hölttä, E. and Pohjanpelto, P. (1986) *J. Biol. Chem.* **261**, 9502–9508
- 61 Utsunomiya, K., Kanamoto, R. and Hayashi, S. (1985) *Jikeikai Med. J.* **32**, 575–585
- 62 Pösö, H., Kallio, A., Scalabrino, G. and Jänne, J. (1977) *Biochim. Biophys. Acta* **497**, 288–297
- 63 Pegg, A. E., Conover, C. and Wrona, A. (1978) *Biochem. J.* **170**, 651–660
- 64 Fong, W. F., Heller, J. S. and Canellakis, E. S. (1976) *Biochim. Biophys. Acta* **428**, 456–465
- 65 Heller, J. S., Fong, W. F. and Canellakis, E. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1858–1862
- 66 Baby, T. G. and Hayashi, S. (1991) *Biochim. Biophys. Acta* **1092**, 161–164
- 67 Baby, T. G. and Hayashi, S. (1991) *Comp. Biochem. Physiol.* **99B**, 151–156
- 68 Kitani, T. and Fujisawa, H. (1984) *J. Biol. Chem.* **259**, 10036–10040
- 69 Fujita, K., Murakami, Y. and Hayashi, S. (1982) *Biochem. J.* **204**, 647–652
- 70 Flamigni, F., Stefanelli, C., Guarnieri, C. and Calderara, C. M. (1986) *Biochim. Biophys. Acta* **882**, 377–383
- 71 Murakami, Y. and Hayashi, S. (1985) *Biochem. J.* **226**, 893–896
- 72 Matsufuji, S., Kanamoto, R., Murakami, Y. and Hayashi, S. (1990) *J. Biochem. (Tokyo)* **107**, 87–91
- 73 Matsufuji, S., Miyazaki, Y., Kanamoto, R., Kameji, T., Murakami, Y., Baby, T. G., Fujita, K., Ohno, T. and Hayashi, S. (1990) *J. Biochem. (Tokyo)* **108**, 365–371
- 74 Li, X. and Coffino, P. (1994) *Mol. Cell. Biol.* **14**, 87–92
- 75 Ichiba, T., Matsufuji, S., Miyazaki, Y., Murakami, Y., Tanaka, K., Ichihara, A. and Hayashi, S. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1721–1727
- 76 Mitchell, J. L. A., Judo, G. G., Bareyal-Leyser, A. and Ling, S. Y. (1994) *Biochem. J.* **299**, 19–22
- 77 Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S. and Igarashi, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8930–8934
- 78 Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F. and Hayashi, S. (1994) *Cell*, in the press
- 79 Miyazaki, Y., Matsufuji, S. and Hayashi, S. (1992) *Gene* **113**, 191–197
- 80 Matsufuji, S., Miyazaki, Y., Murakami, Y., Matsufuji, T., Atkins, J. F., Gesteland, R. F. and Hayashi, S. (1993) in *Workshop on Alternate Readings of the Genetic Code*. Proc. HFSPD, EMBO, FEBS, and IUBMB Meeting, p55 (abstr.), Parknasilla, Co. Kerry, Ireland
- 81 Rom, E. and Kahana, C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3959–3963
- 82 Rom, E. and Kahana, C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9195
- 83 Gesteland, R. F., Weiss, R. B. and Atkins, J. F. (1992) *Science* **257**, 1640–1641
- 84 Atkins, J. F. and Gesteland, R. F. (1995) in *tRNA: Structure, Biosynthesis and Functions* (Söll, D. and RajuBhandary, U. L., eds.), pp. 471–490, Am. Soc. Microbiol., Washington, DC
- 85 Craigen, W. J., Cook, R. G., Tate, W. P. and Caskey, C. T. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3616–3620
- 86 Pojanpelto, P. (1976) *Exp. Cell Res.* **102**, 138–142
- 87 Mitchell, J. L. A., Diveley, R. R., Jr. and Bareyal-Leyser, A. (1992) *Biochem. Biophys. Res. Commun.* **186**, 81–88
- 88 Heller, J. S., Kyriakidis, D. A., Fong, W. F. and Canellakis, E. S. (1977) *Eur. J. Biochem.* **81**, 545–550
- 89 Murakami, Y., Marumo, M. and Hayashi, S. (1986) *FEBS Lett.* **199**, 49–52
- 90 Isekson, I. and Kaye, A. M. (1976) *FEBS Lett.* **61**, 54–58
- 91 Murakami, Y., Kameji, T. and Hayashi, S. (1984) *Biochem. J.* **217**, 573–580
- 92 Zuretti, M. F., Brossa, O., Gili, P. and Gravela, E. (1988) *Cell Biochem. Funct.* **6**, 107–114
- 93 Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A. and Kahana, C. (1989) *J. Biol. Chem.* **264**, 15949–15952
- 94 Rosenberg-Hasson, Y., Berkovich, Z., Ciechanover, A. and Kahana, C. (1989) *Eur. J. Biochem.* **185**, 469–474
- 95 Choi, J. H. and Scheffler, I. E. (1983) *J. Biol. Chem.* **258**, 12601–12607
- 96 Kanamoto, R., Kameji, T., Iwashita, S., Igarashi, K. and Hayashi, S. (1993) *J. Biol. Chem.* **268**, 9393–9399
- 97 Murakami, Y., Matsufuji, S., Tanaka, K., Ichihara, A. and Hayashi, S. (1993) *Biochem. J.* **295**, 305–308
- 98 Bond, J. S. and Butler, P. E. (1987) *Annu. Rev. Biochem.* **56**, 333–364
- 99 Haas, A. L. and Rose, I. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6845–6848
- 100 Goldberg, A. L. (1992) *Eur. J. Biochem.* **203**, 9–23
- 101 Hershko, A. and Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61**, 761–807
- 102 Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, A. (1992) *New Biologist* **4**, 173–187
- 103 Rivett, A. J. (1993) *Biochem. J.* **291**, 1–10
- 104 Ugai, S.-I., Tamura, T., Tanahashi, N., Takai, S., Komi, N., Chung, C. H., Tanaka, K. and Ichihara, A. (1993) *J. Biochem. (Tokyo)* **113**, 754–768
- 105 Ciechanover, A. and Schwartz, A. L. (1994) *FASEB J.* **8**, 188–191
- 106 Mamroud-Kidron, E., Rosenberg-Hasson, Y., Rom, E. and Kahana, C. (1994) *FEBS Lett.* **337**, 239–242
- 107 Phillips, M. A., Coffino, P. and Wang, C. C. (1987) *J. Biol. Chem.* **262**, 8721–8727
- 108 Bacchi, C. J., Nathan, H. C., Hutner, S. H., McCann, P. P. and Sjoerdsma, A. (1980) *Science* **210**, 332–334
- 109 Sjoerdsma, A. and Schechter, P. J. (1984) *Clin. Pharmacol. Ther.* **35**, 287–298
- 110 Ghoda, L., Phillips, M. A., Bass, K. E., Wang, C. C. and Coffino, P. (1990) *J. Biol. Chem.* **265**, 11823–11826
- 111 Macrae, M. and Coffino, P. (1987) *Mol. Cell. Biol.* **7**, 564–567
- 112 Rogers, S., Wells, R. and Rechsteiner, M. (1986) *Science* **234**, 364–368
- 113 Rechsteiner, M. (1988) *Adv. Enzyme Regul.* **27**, 135–151
- 114 Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D. and Coffino, P. (1989) *Science* **243**, 1493–1495
- 115 Lu, L., Stanley, B. A. and Pegg, A. E. (1991) *Biochem. J.* **277**, 671–675
- 116 Rosenberg-Hasson, Y., Bercovich, Z. and Kahana, C. (1991) *Eur. J. Biochem.* **196**, 647–651
- 117 Loetscher, P., Pratt, G. and Rechsteiner, M. (1991) *J. Biol. Chem.* **266**, 11213–11220
- 118 Miyazaki, Y., Matsufuji, S., Murakami, Y. and Hayashi, S. (1993) *Eur. J. Biochem.* **214**, 837–844
- 119 Ghoda, L., Sidney, D., Macrae, M. and Coffino, P. (1992) *Mol. Cell. Biol.* **12**, 2178–2185
- 120 Gupta, M. and Coffino, P. (1985) *J. Biol. Chem.* **260**, 2941–2944
- 121 Kahana, C. and Nathans, D. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1673–1677
- 122 Tobias, K. E. and Kahana, C. (1993) *Biochemistry* **32**, 5842–5847
- 123 Coleman, C. S., Stanley, B. A., Viswanath, R. and Pegg, A. E. (1994) *J. Biol. Chem.* **269**, 3155–3158
- 124 Solano, F., Peñafiel, R., Solano, M. E. and Losano, J. A. (1985) *FEBS Lett.* **190**, 324–328
- 125 Mitchell, J. L. A., Lynning, M. D., Hong, H. J. and Hicks, H. F. (1988) *Arch. Biochem. Biophys.* **264**, 585–594
- 126 Mitchell, J. C. A. and Chen, H. J. (1990) *Biochim. Biophys. Acta* **1037**, 115–121
- 127 Rosenberg-Hasson, Y., Bercovitz, Z. and Kahana, C. (1991) *Biochem. J.* **277**, 683–685
- 128 Tobias, K. E., Mamroud-Kidron, E. and Kahana, C. (1993) *Eur. J. Biochem.* **218**, 245–250
- 129 Tokunaga, F., Goto, T., Koide, T., Murakami, Y., Hayashi, S., Tamura, T., Tanaka, K. and Ichihara, A. (1994) *J. Biol. Chem.* **269**, 17382–17385
- 130 Goldberg, L. A. and Rock, K. L. (1992) *Nature (London)* **357**, 375–379
- 131 Monako, J. J. (1992) *Immunol. Today* **13**, 173–179
- 132 Watanabe, S.-I., Kusama-Eguchi, K., Kobayashi, H. and Igarashi, K. (1991) *J. Biol. Chem.* **266**, 20803–20809
- 133 Grillo, M. A., Bedino, S. and Testore, G. (1980) *Int. J. Biochem.* **11**, 37–42
- 134 Fonzi, W. A. and Sypherd, P. S. (1987) *J. Biol. Chem.* **262**, 10127–10133
- 135 Fonzi, W. A. (1989) *J. Biol. Chem.* **264**, 18110–18118

- 136 Davis, R. H., Krasner, G. N., DiGangi, J. J. and Ristow, J. L. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4105–4109
- 137 Williams, L. J., Barnett, G. R., Ristow, J. L., Pitkin, J., Perriere, M. and Davis, R. H. (1992) *Mol. Cell. Biol.* **12**, 347–359
- 138 Assaraf, Y. G., Kahana, C., Spira, D. T. and Bachrach, U. (1988) *Exp. Parasitol.* **67**, 20–30
- 139 Ceriani, C., González, N. S. and Algranati, I. D. (1992) *FEBS Lett.* **301**, 261–264
- 140 DiGangi, J. J., Seyfzadeh, M. and Davis, R. H. (1987) *J. Biol. Chem.* **262**, 7889–7893
- 141 Hanson, S., Adelman, J. and Ullman, B. (1992) *J. Biol. Chem.* **267**, 2250–2259
- 142 Mitchell, J. L. A., Augustine, T. A. and Wilson, J. M. (1981) *Biochim. Biophys. Acta* **657**, 257–267
- 143 Mitchell, J. L. A. (1983) *Methods Enzymol.* **94**, 140–146
- 144 Heller, J. S., Kyriakidis, D. A. and Canellakis, E. S. (1983) *Biochim. Biophys. Acta* **760**, 154–162
- 145 Panagiotidis, C. A. and Canellakis, E. S. (1984) *J. Biol. Chem.* **259**, 15025–15027
- 146 Huang, S.-C., Panagiotidis, C. A. and Canellakis, E. S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3464–3468
- 147 Canellakis, E. S., Paterakis, A. A., Huang, S.-C., Panagiotidis, C. A. and Kyriakidis, D. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7129–7133
- 148 Koromilas, A. E. and Kyriakidis, D. A. (1987) *Plant Growth Regulation* **6**, 267–275
- 149 Hiatt, A. C., McIndoo, J. and Malmberg, R. L. (1986) *J. Biol. Chem.* **261**, 1293–1298
- 150 Bishop, P. B., Young, J., Peng, T. and Richards, J. F. (1985) *Biochem. J.* **226**, 105–112
- 151 Hu, Y.-W. and Brosnan, M. E. (1987) *Arch. Biochem. Biophys.* **254**, 637–641
- 152 Richards, J. F., Bishop, P. B., Peng, T., Beer, C. T. and Gout, P. W. (1986) *Biochim. Biophys. Acta* **883**, 542–551
- 153 Kitani, T. and Fujisawa, H. (1989) *Biochim. Biophys. Acta* **991**, 44–49
- 154 Murakami, Y., Nishiyama, M. and Hayashi, S. (1989) *Eur. J. Biochem.* **180**, 181–184
- 155 van Daalen-Wetters, T., Macrae, M., Brabant, M., Sittler, A. and Coffino, P. (1989) *Mol. Cell. Biol.* **9**, 5484–5490
- 156 Persson, L., Holm, I. and Heby, O. (1988) *J. Biol. Chem.* **263**, 3528–3533
- 157 Murakami, Y., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1994) *Biochem. J.* **304**, 183–187