REVIEW ARTICLE

The proteasome activator 11 S REG (PA28) and Class I antigen presentation

Martin RECHSTEINER[1], Claudio REALINI[2] and Vicença USTRELL

Department of Biochemistry, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, UT 84132, U.S.A.

There are two immune responses in vertebrates: humoral immunity is mediated by circulating antibodies, whereas cytotoxic T lymphocytes (CTL) confer cellular immunity. CTL lyse infected cells upon recognition of cell-surface MHC Class I molecules complexed with foreign peptides. The displayed peptides are produced in the cytosol by degradation of host proteins or proteins from intracellular pathogens that might be present. Proteasomes are cylindrical multisubunit proteases that generate many of the peptides eventually transferred to the cell surface for immune surveillance. In mammalian proteasomes, six active sites face a central chamber. As this chamber is sealed off from the enzyme's surface, there must be mechanisms to promote entry of substrates. Two protein complexes have been found to bind the ends of the proteasome and activate it. One of the activators is the 19 S regulatory complex of the 26 S proteasome; the other activator is '11 S REG' [Dubiel, Pratt, Ferrell and Rechsteiner (1992) J. Biol. Chem. **267**, 22369–22377] or 'PA28' [Ma, Slaughter and DeMartino (1992) J. Biol. Chem. **267**, 10515–10523]. During the past 7 years, our understanding of the structure of REG molecules has increased significantly, but much less is known about their biological functions. There are three REG subunits, namely α, β and γ. Recombinant REGα forms a ring-shaped heptamer of known crystal structure. 11 S REG is a heteroheptamer of α and β subunits. REGγ is also presumably a heptameric ring, and it is found in the nuclei of the nematode work *Caenorhabditis elegans* and higher organisms, where it may couple proteasomes to other nuclear components. REGα and REGβ, which are abundant in vertebrate immune tissues, are located mostly in the cytoplasm. Synthesis of REG α and β subunits is induced by interferon-γ, and this has led to the prevalent hypothesis that REG α/β hetero-oligomers play an important role in Class I antigen presentation. In the present review we focus on the structural properties of REG molecules and on the evidence that REGα/β functions in the Class I immune response.

Key words: cellular immunity, 26 S proteasome, protein degradation, ubiquitin-mediated proteolysis.

INTRODUCTION

Proteolysis serves to maintain the structural and metabolic integrity of cells in several ways. Proteins with altered conformation due to mutation, denaturation or premature chain termination are rapidly degraded [1]. Intracellular proteolysis also plays a key role in protein targeting. Leader and transit peptides are degraded after proteins reach their proper compartments [2,3]. Self-assembly of multiprotein complexes is also accompanied by proteolysis, since cells generally destroy any excess subunits [4]. In addition to its role in self-assembly, proteolysis serves as an important regulatory mechanism. Maximal rates at which protein levels can change are determined by the protein's half-life [5], and key metabolic enzymes are often rapidly degraded [6]. Non-enzymic proteins can also be short-lived, and such proteins are usually components of important regulatory pathways, e.g. Myc, Fos, p53, etc. Proteolysis appears to be particularly important in controlling orderly transitions during the cell cycle [7]. Finally, degradation of cytoplasmic proteins plays a crucial role in cell-mediated immunity by providing peptides for display on the surfaces of virally infected cells [8].

Intracellular pathogens pose a special problem for the immune system because they are separated from circulating antibodies by the infected cell's plasma membrane. This is particularly dangerous when the pathogen spreads directly to neighbouring cells, as is the case with a number of viruses and even some bacteria [9]. Vertebrates have developed an elegant system for defending themselves against intracellular pathogens, principally viruses. Following infection, newly synthesized viral proteins are reduced to small fragments in the host-cell cytoplasm, and the resulting peptides are displayed on the plasma membrane bound to MHC Class I molecules. These surface-exposed MHC–peptide complexes are recognized by a specific class of lymphocytes, known as cytotoxic T lymphocytes (CTL), and the infected cells are lysed by several different mechanisms [10]. Viral proteins taken up by phagocytosis are also degraded within endosomal compartments to peptide fragments that are displayed on the cell surface. In this case, however, the peptides associate with MHC Class II molecules and are recognized by helper T lymphocytes rather than CTL [11]. Helper T cells promote antibody production rather than kill infected cells. Class II antigen presentation is, therefore, distinct from the Class I pathway, and it is not covered in the present review.

Abbreviations used: CTL, cytotoxic T lymphocytes; TAPs, transporters associated with antigen presentation; ER, endoplasmic reticulum; T-L, trypsin-like; CT(-L), chymotrypsin(-like); PGPH, peptidylglutamyl-preferring; BrAAP, branched-chain-preferring; SNAAP, small-neutral-preferring; GTT, single-letter code for Gly-Thr-Thr; ^{125}I-Tyr-Gly-Arg-CH_2Cl, ^{125}I-Tyr-Gly-Arg-chloromethane; IFN(γ), interferon(-γ); Δi, deletion of homologue-specific inserts; N146Y etc., Asn^{146} $\rightarrow$ tyrosine etc.

[1] To whom correspondence should be addressed (e-mail rechsteiner@mailman.med.utah.edu).

[2] Present Address: Oncology Institute of Southern Switzerland, Laboratory of Experimental Oncology, Ospedale La Carita, Via all'Ospedale 1, 6600 Locarno, Switzerland.

The mechanism by which viral peptides are generated in the cytosol and transferred to the cell surface has been the subject of intense research for more than a decade, and today we know, in broad outline at least, how most peptides are presented on Class I molecules. There is good evidence that proteasomes generate many Class I peptides: the interferon (IFN)-inducible proteasome subunits LMP2 and LMP7 are encoded in the MHC [12], and the reasonably specific proteasome inhibitor lactacystin markedly reduces Class I presentation [13,14]. The newly formed peptides enter the endoplasmic reticulum (ER) through peptide transporters associated with antigen presentation (TAPs). Once inside the ER, the peptides bind to MHC Class I molecules, which are then released from ER chaperonins, e.g., tapasins, calnexin and calsequestrin [15], and the MHC I–peptide complexes are transferred to the plasma membrane by normal secretory processes.

The 20 S proteasome, by itself, does not degrade intact proteins. To do so, the enzyme must associate with a regulatory complex that contains 18 different subunits, among which are six ATPases. The combination of 20 S proteasome and 19 S regulatory complex produces the 26 S proteasome, which is responsible for the ATP-dependent degradation of many cellular proteins, especially those marked for destruction by polyubiquitin chains [1,16]. The 20 S proteasome also binds ring-shaped molecules known as REG or PA28. Although the REG greatly stimulates the proteasome's peptidase activities, REG–proteasome complexes are not capable of degrading intact proteins. In addition to free 20 S proteasomes, REG–proteasome complexes and the 26 S proteasome, Hendil et al. [17] have recently described a species of 20 S proteasome containing a REG ring at one end and a 19 S regulatory complex at the other. It is not known which of these proteasomes is primarily responsible for generating peptides destined for Class I presentation. In the present review we discuss the 11 S REG and its presumed role in generating antigenic peptides. A number of recent reviews offer broader treatments of Class I antigen presentation [18–22].

20 S PROTEASOMES

Subunit composition

The 20 S proteasome is a major intracellular proteolytic complex found in archaebacteria, eubacteria and eukaryotes. The enzyme consists of 28 subunits arranged as four rings of seven subunits each [23]. The rings stack upon one another to form a cylindrical particle that measures 10 nm in diameter by 15 nm in length [24,25]. The proteasome from the archaebacterium *Thermoplasma acidophilum* is constructed from multiple copies of two unique subunits, called α and β [26]. The catalytically inactive α subunits comprise the end rings, and proteolytic β subunits form the two central rings. cDNAs that encode proteasome subunits have been sequenced from a wide variety of organisms [27], and the subunits can be grouped into α and β families. The subunit composition of eukaryotic proteasomes is more complicated than that of the archaebacterial enzyme. Seven distinct α subunits and at least ten distinct β subunits have been described in vertebrates [28]. Cross-linking studies and electron-microscopic analyses of antibody-decorated human proteasomes [29], as well as X-ray diffraction of the yeast proteasome [25], reveal that the seven unique α subunits occupy defined positions within an α ring. The seven yeast β subunits are also arranged in a fixed order ([25]; see the present Figure 1).

The *Thermoplasma* proteasome, with 14 copies of the same β subunit [26], preferentially hydrolyses small fluorogenic peptides with hydrophobic residues in the P1 position. For this reason, it is said to exhibit chymotrypsin-like activity [30]. Eukaryotic proteasomes cleave a wider variety of fluorogenic peptides. Soon after their initial description of the multicatalytic protease (proteasome), Wilk and Orlowski [31] used inhibitors to identify trypsin-like (T-L), chymotrypsin-like (CT-L), and peptidyl-glutamyl-preferring (PGPH) catalytic activities in the pituitary enzyme. Subsequent studies identified two additional proteolytic activities, the branched-chain-preferring (BrAAP) and small-neutral-preferring (SNAAP) sites [32]. Yeast proteasomes with mutations in β subunits confirm that individual subunits are largely responsible for the hydrolysis of specific fluorogenic peptides [33–37].

Catalytic mechanism

The proteasome uses a threonine residue for nucleophilic attack on the carbonyl moiety within a peptide bond. This has been shown in several ways. Baumeister and his colleagues employed site-directed mutagenesis to probe potential catalytic residues in the *Thermoplasma* β subunit and found that mutation of the N-terminal threonine leads to folded, but inactive, subunits [38]. Fenteany et al. [39] demonstrated that lactacystin, a bacterial metabolite that inhibits proteasome activity, forms covalent adducts to the newly formed N-terminal threonine residue of a human β proteasome subunit. Moreover, the crystal structure of the *Thermoplasma* proteasome bound to acetyl-Leu-Leu-norleucinal places the protease inhibitor very near the newly generated N-terminal threonine residues of the β subunits [40].

Thermoplasma β subunits provide each enzyme particle with 14 active sites. There are seven β subunits common to all eukaryotic proteasomes, but only three contain the Gly-Thr-Thr (GTT) processing site that generates the catalytically important N-terminal threonine residue in the *Thermoplasma* enzyme. This observation led Seemüller et al. [38] to propose that eukaryotic proteasomes contain three active, and four inactive, β subunits. If this is correct, two active β subunits, bearing the PGPH and T-L sites, sit adjacent to each other, with the subunit responsible for CT-L activity being surrounded by inactive subunits (see Figure 1). Two human β subunits that lack the GTT processing site, C5 and N3, are processed eight or nine residues N-terminal to a threonine residue similar to the N-terminal threonine in the other subunits [41]. If the catalytic threonine need not be at the N-terminus, then these subunits could be active. It is even possible that some β subunits lacking the requisite threonine may be proteolytically active. In this regard, Rivett and her colleagues [42] have observed labelling of rat β subunit C7 by the active-site inhibitor ^{125}I-Tyr-Gly-Arg-chloromethane (^{125}I-Tyr-Gly-Arg-CH_2Cl), despite the fact that C7 lacks the N-terminal threonine residue present in active β subunits. This observation coupled with the existence of five kinetically distinct activities in eukaryotic proteasomes leaves open the possibility that higher eukaryotic proteasomes may contain more than three active β subunits.

C-terminal extensions

As mentioned, eukaryotic proteasomes contain a number of different α and β subunits. Comparison of archaebacterial and eukaryotic proteasome sequences reveals the presence of C-terminal extensions on four α subunits and one β subunit from eukaryotic proteasomes (see Figure 1). All four α subunit extensions are highly charged. The extensions on α subunits C6, C8 and C9 are predicted to be α-helical; it is unlikely that the proline-rich extension on C2 can form an α helix. The extensions on subunits C6 and C9 consist of 'alternating' lysine (K) and glutamate (E) residues. These 'KEKE motifs' are particularly interesting because similar tracts of 'alternating' glutamate and

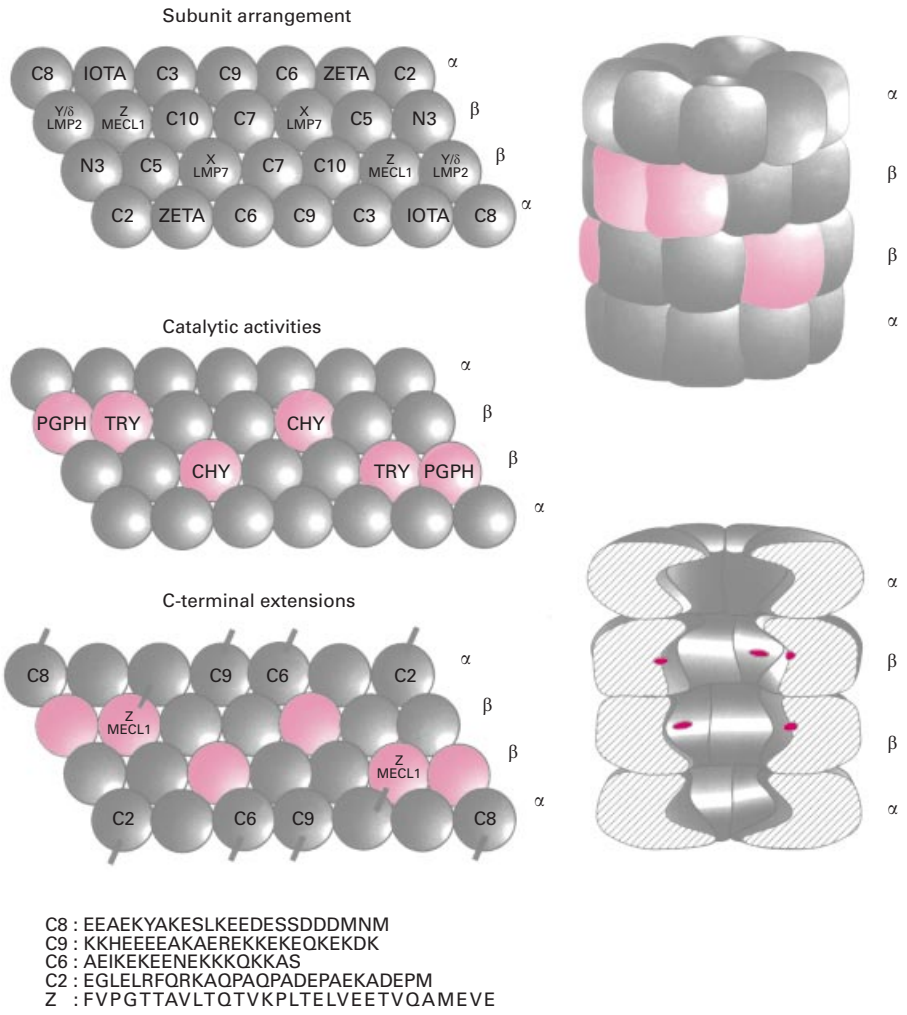

Figure 1 The human 20 S proteasome

The three panels at the left depict the subunit arrangement, catalytic activities and C-terminal extensions of proteasome subunits. The α's and β's to the right identify the α and β rings of the proteasome. TRY and CHY are subunits that exhibit T-L and CT-L activities respectively. The assembled 20 S proteasomes are depicted at the right. The lower 'cutaway' diagram highlights the internal chambers. The spheres coloured pink represent the proteolytically active β subunits and the small red ellipses in the cutaway diagram represent the active sites.

lysine residues are present in proteins that associate with the proteasome, namely REGα (or PA28α), as well as five subunits in the regulatory complex of the 26 S proteasome. KEKE motifs are also found in various chaperonins, including heat-shock protein 70 ('hsp90'), which associates with the proteasome. It has been hypothesized that KEKE sequences mediate protein–protein interactions [43]. This idea is supported by recent observations that regions containing KEKE motifs in the calcium-release channel and dihydropyridine receptors promote their mutual interactions [44].

Quarternary structure and the 'molecular-ruler' hypothesis

Crystal structures of the archaebacterial and yeast proteasomes have revealed three chambers within the particle. Two chambers are formed by α and β rings, and a somewhat larger central chamber is encompassed by the two β rings (see Figure 1). The central proteolytic chamber has a volume equivalent to a 70 kDa globular protein [$\approx$ 80000 Å^3 (1 Å = 0.1 nm)] . It connects with each α/β chamber through pores or 'gates' about 20 Å in diameter. The 14 active sites in the central chamber of the archaebacterial proteasome are spaced almost 30 Å apart. This spacing is relevant to an idea known as the 'molecular ruler' hypothesis. On the basis of their analysis of peptides generated from haemoglobin and the insulin B chain by the *Thermoplasma* proteasome, Wenzel et al. [45] proposed that proteasomes cleave polypeptide chains every eight to ten amino acids. Since ten residues in an extended β conformation are approx. 30 Å in length, the distribution of active sites in the archaebacterial enzyme would appear ideal for producing peptides that long. The molecular-ruler hypothesis is also attractive from an immunological perspective, because MHC Class I epitopes are almost always eight to eleven amino acids long. Unfortunately, there are problems with the concept that proteasomes preferentially cleave polypeptide chains every eight to ten residues. If there are only three active β subunits in the higher-eukaryotic proteasome, then active sites are not always spaced at 30 Å intervals. Another problem is that one must invoke some mechanism to force polypeptide chains into fully extended β conformations. Wang et al. [46] recently solved the crystal structure of ClpP, a prokaryotic

protease that, like the proteasome, contains subunits arranged in two heptameric rings. They suggest that a hydrophobic groove linking the ClpP active sites serves as a continuous substrate-binding surface capable of producing stretches of β strand. However, it is not clear how such a hydrophobic groove could generate β strands, since hydrophobic and charged residues are extensively intermingled in most natural substrates.

Several recent publications provide experimental evidence against the molecular-ruler hypothesis. Kisselev et al. [47] analysed peptides produced by the *Thermoplasma* proteasome and found that the average size of the degradation products was substrate-dependent. Although peptides formed from lactalbumin and casein averaged eight and eleven residues respectively, the mean size of peptides derived from alkaline phosphatase or insulin-like growth factor was only six residues. Moreover, individual peptides ranged from three residues to 30 residues, and peptide lengths formed a log normal distribution. From these studies and similar analyses using mammalian 26 S and 20 S proteasomes [48], Kisselev et al. proposed that proteolysis continues until products are small enough to diffuse out of the proteasome. Dolenc et al. [49] arrived at a similar conclusion upon finding that the archaebacterial proteasome degraded peptides longer than 14 residues faster than shorter peptides. Studies using mutant yeast proteasomes cast further doubt on the molecular-ruler hypothesis. Nussbaum et al. [50] found that degradation of enolase by wild-type yeast proteasomes and yeast proteasomes lacking CT, T or PGPH active sites produced very similar peptide length distributions. They concluded that fragment length is not influenced by the distance between active sites, and reinforced this conclusion in another study in which cleavage of fluorogenic and natural peptides was analysed using wild-type and mutant yeast proteasomes [51]. Once again, the average fragment length produced by digestion of natural peptides did not differ between wild-type and mutant proteasomes.

Substrate access

It is evident from crystal structures that access to the proteasome's internal chambers is greatly restricted. There is a pore approx. 13 Å in diameter through each α ring leading to the α/β chambers of the archaebacterial proteasome (see Figure 1). The α/β chambers of the yeast proteasome are virtually inaccessible from the particle's surface because N-terminal sequences in α subunits interact extensively, forming a seal at each end of the cylinder [25]. Channels that connect the interior antechambers to the lateral surface of the yeast proteasome are present between the α and β rings. But they are only 10 Å in diameter, probably too small to allow significant amounts of peptide into or out of the particle. Substrates must enter the proteasome to be cleaved, so there must be some mechanism to produce openings to the internal chambers. Two particles have been discovered that bind the proteasome and activate peptide cleavage. One is a ring-shaped multimer called the 11 S REG or PA28 [52,53]. The other is a large multisubunit assembly [54–57], called the 19 S regulatory complex (or PA700), that is a component of an even larger 26 S protease. A major proteolytic pathway in eukaryotes involves the covalent attachment of the small highly conserved protein, ubiquitin, to substrate proteins [1]. With a few exceptions, this modification targets proteins for degradation by the ATP-dependent 26 S proteasome [58]. Because there is evidence that ubiquitin-mediated proteolysis can be important for Class I antigen presentation, we briefly describe the 19 S regulatory complex. Extensive discussion of such a complicated particle is beyond the scope of the present review.

The 19 S regulatory complex

The 19 S regulatory complex is best discussed in the context of the larger 26 S proteasome, which was discovered in 1986 [59]. Following its purification a year later, the 26 S proteasome was found to contain more than 30 different subunits [60]. On the basis of its subunit composition and published electron micrographs of a 26 S mushroom-shaped particle [61], a model of the enzyme was proposed in which the 'cylindrical' proteasome is attached to a 'spherical' particle (the 19 S regulatory complex). The regulatory complex was postulated to confer substrate recognition and energy-dependence for the degradation of ubiquitin conjugates [62]. Support for this model was obtained when several groups found that the 26 S proteasome can be assembled from two protein complexes, namely the proteasome and a 'spherical' 19 S regulatory complex [54–57]. Electron-microscopic studies [63,64] have produced both mushroom-shaped and barbell-shaped images of the 26 S protease, indicating that the proteasome can be capped by one or two regulatory complexes.

cDNAs have been isolated and sequenced for all of the subunits in the regulatory complex [65]. Six subunits contain motifs indicating that they are ATPases [66], but only two of the non-ATPase subunits have been assigned functions. Subunit 5a (S5a) was identified by its ability to bind polyubiquitin chains [67]. Although S5a is thought to play a role in substrate recognition, deletion of the gene encoding the yeast subunit is not lethal [68]. A second, 38 kDa subunit has been reported to 'edit' ubiquitin chains [69]. The remaining subunits are presumably involved in substrate recognition, may confer structural integrity to the regulatory complex or serve to localize the 26 S proteasome within cells.

The location of specific subunits within the regulatory complex is an important unanswered question. The regulatory complex exhibits ATPase activity [57,70], and the expended energy is probably used to unfold and transfer protein substrates into the proteasome. Individual ATPases have been shown to associate in pairs or tetramers [71], making it likely that the six proteins sit next to one another within the regulatory complex. It is reasonable to believe that the ATPases directly contact the proteasome α rings in the assembled 26 S proteasome, since this arrangement would place the presumed peptide pumps directly over the internal chambers of the proteasome. In fact, recent studies demonstrate that the six ATPases and the two largest regulatory-complex subunits remain attached to the 20 S proteasome upon high-salt treatment of yeast 26 S proteasomes that are fragile because they contain a mutant S5a subunit [72].

THE 11 S REG OR PA28

Formation of the 26 S proteasome from the regulatory complex and the 20 S proteasome produces a modest increase in the latter's peptidase activities [73]. A second activator was independently discovered by four groups in the early 1990s [52,53,74,75]. It increases proteasome-mediated hydrolysis of some fluorogenic peptides as much as 100-fold, whereas cleavage of other peptides can be unaffected [76]. The activator increases V_{max} and reduces K_m for hydrolysis of peptides by the proteasome, but it does not confer upon the 20 S proteasome the ability to degrade intact proteins or ubiquitin conjugates [52,53]. As isolated from red blood cells, the factor sediments at 11 S and is composed of two subunits with apparent molecular masses of 30 kDa [53]). Electron-microscopic studies have shown that the subunits form rings that bind one or both ends of the proteasome [77]. Binding is fully reversible, and the activator is not modified

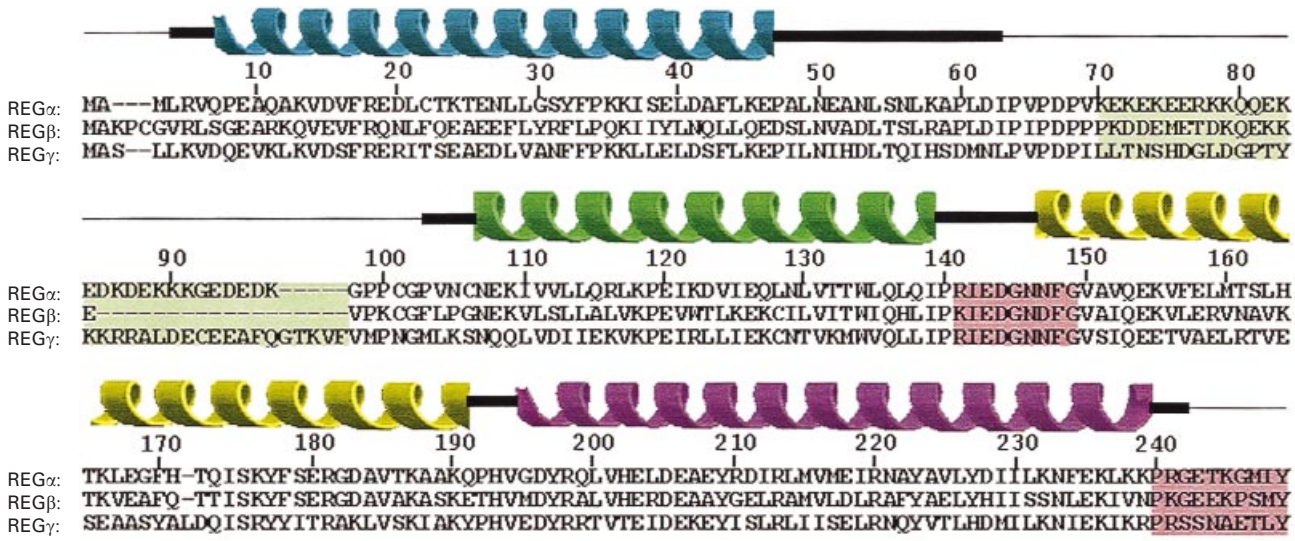

Figure 2 REGα secondary structure and sequence alignment with human REGβ and REGγ

Helices are indicated as follows: helix 1 (blue; residues 7–46), helix 2 (green; 107–139), helix 3 (yellow; 147–191), and helix 4 (magenta; 195–239). Helix 1 is kinked by $\approx 45°$ at Pro^{34}, and helix 4 has a severe $\approx 65°$ kink at Phe^{234}. The 48 residues that lack electron density (1–3, 64–102 and 243–249) have been omitted from the model and are indicated by a thin line. The subunit-specific inserts (70–97) are shown against a green background. Sequences implicated in binding and activation of the proteasome (141–149 and 240–249) are shown on a dark pink background. Reprinted with permission from Nature [92] © 1997 Macmillan Magazines Limited.

by its association with the proteasome [53]. We call this proteasome activator '11 S REG' [53]; DeMartino and his colleagues designate it 'PA28' [52].

Red-blood-cell REG is composed of two subunits, REGα and REGβ [53]. Isolation of a cDNA for human REGα [78] revealed the subunit to be identical with a protein induced by IFNγ treatment of human keratinocytes [79]. Subsequently, cDNAs for human REGβ have been isolated [80], and today the REG subunit sequences are available from various organisms. Human REGα and REGβ share extensive sequence identity, and they are related to another protein, Ki, discovered as a major autoantigen in patients suffering from lupus erythematosus [81]. Because recombinant Ki activates the proteasome [82], we refer to it as 'REGγ'. Amino acid sequences of the three REG homologues are presented in Figure 2, with two regions highlighted. Short stretches of 16–32 amino acids that diverge considerably among the three proteins are against a green background. These divergent sequences are called homologue-specific 'inserts', although there is no evidence for actual insertion during evolution. Sequences most highly conserved among the three REG homologues are highlighted in dark pink. As discussed below, one of these conserved regions contains nine amino acids that form a proteasome activation loop.

Biochemical properties of recombinant REG subunits

Each human REG homologue has been produced in *Escherichia coli* and characterized [82]. REGα and REGγ form heptamers that activate the proteasome at submicromolar concentrations. There are, however, distinct differences in the patterns of activation. REGγ stimulates hydrolysis of peptides with basic residues next to the fluorescent leaving group. It is a much less potent activator when fluorogenic peptides with acidic or hydrophobic residues in the P1 position are used as substrates. By contrast, REGα activates cleavage after basic, acidic and many hydrophobic residues [82]. REGγ binds the proteasome with higher affinity than REGα; both bind less tightly than REGα/REGβ hetero-oligomers. Recombinant REGβ subunits chromatograph as monomers upon gel filtration and form hetero-oligomers when mixed with REGα [82]. Whether REGβ subunits activate the proteasome is controversial. Human REGβ was reported to be inactive [83]; recombinant rat REGβ was also reported incapable of activating the proteasome [84]. However, three studies from our laboratory have shown that REGβ, at micromolar concentrations, stimulates fluorogenic-peptide hydrolysis by the proteasome in a manner virtually identical with that by REGα [82,85,86]. In one of these studies, a single-site mutation in REGβ resulted in loss of proteasome-stimulating activity [85]. Furthermore, recombinant REGβ stimulates peptide hydrolysis by yeast proteasomes (C. Realini, C. Jensen, S. Endicott, C. Avendt, Z. Zhang, M. Hochstrasser and M. Rechsteiner, unpublished work). This finding eliminates the possibility that REGβ subunits simply augment activation by REGα molecules present in our proteasome preparations, because yeast lacks genes encoding any of the REG subunits. Although the idea that REGβ is inactive appears to be widespread [87], we consider the positive demonstration that REGβ is, by itself, a proteasome activator more compelling than the negative reports cited above.

Quaternary structure of REG

SDS/PAGE analysis of purified human red-blood-cell REG revealed two distinct subunits [53]. Bovine red-cell REG (PA28) was originally reported to consist of a single subunit [52], but subsequent studies demonstrated the presence of the two closely related proteins, PA28α and PA28β [88]. As mentioned above, red-blood-cell REG sediments at 11 S, suggesting an apparent molecular mass of 200 kDa for REG complexes. This, in turn, indicates that REG is either a hexamer or a heptamer of 30 kDa subunits. Song et al. [89] combined subunit-specific antibodies and cross-linking experiments to determine whether PA28α and PA28β formed separate rings or mixed rings and whether the rings were hexamers or heptamers. Anti-REGα and anti-REGβ

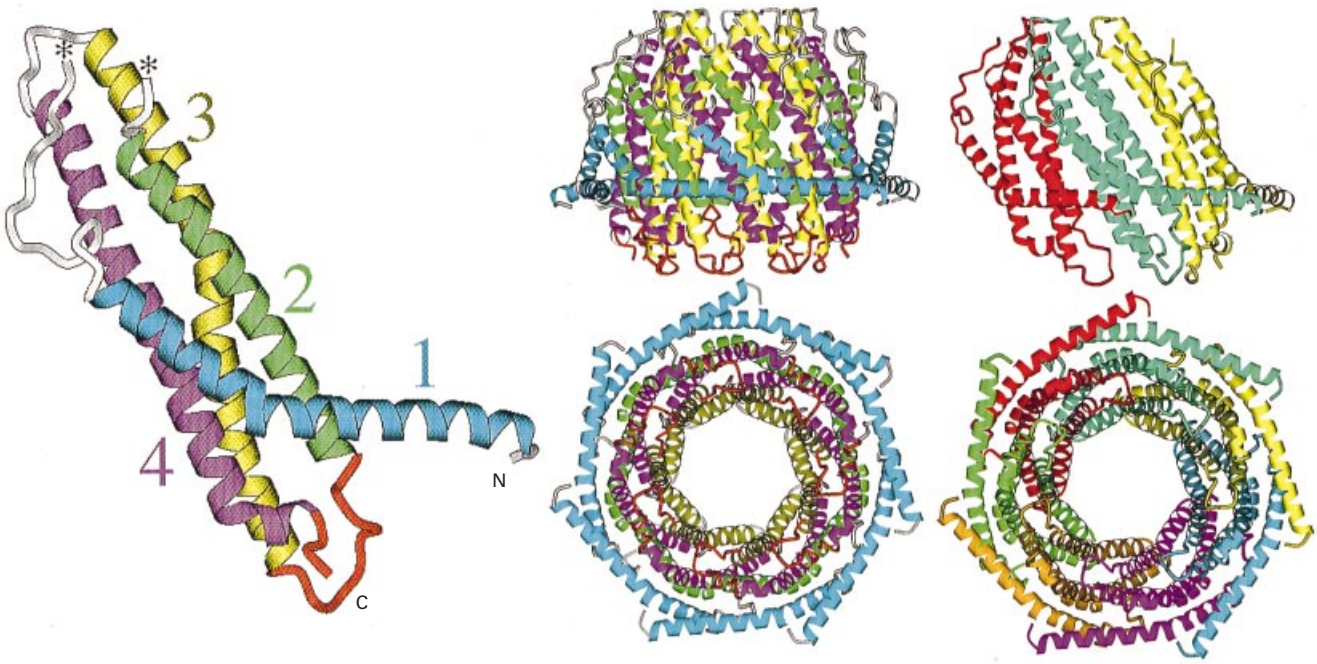

Figure 3 Structure of REGα

Left: ribbon presentation of a REGα monomer coloured with secondary-structural elements. Disordered residues are not modeled in any of the Figures. Colour coding is the same as in Figure 2. The ends of the disordered 39-residue loop are indicated by asterisks. Top centre: the heptamer viewed with the seven-fold axis vertical. Bottom centre: heptamer viewed along the seven-fold axis from underneath that shown in the top-centre panel. Top right: the three subunits nearest the viewer are shown in the same orientation as in the top-centre panel. As shown for the central cyan subunit, each monomer contacts just two other subunits. Bottom right: heptamer coloured with individual monomers and viewed from the same direction as in the bottom centre panel. Reprinted with permission from Nature [92] © 1997 Macmillan Magazines Limited.

antibody staining of cross-linked products separated on SDS/PAGE generated identical patterns, leading Song et al. [89] to conclude that α and β subunits are present in the same ring and that the rings are hexamers with a stoichiometry of $(\alpha\beta)_3$. This conclusion is consistent with studies by Ahn et al. [90], who immunoprecipitated 11 S REG from cells labelled with [^{35}S]methionine, and, on the basis of the relative amounts of radioisotope in REGα and REGβ subunits, they deduced that the 11 S REG is a hexamer containing three α and three β subunits.

The behavior of recombinant REG proteins strongly supports the idea that α and β subunits are present in the same ring. Direct binding assays demonstrated that: (1) REGγ binds only to itself; (2) REGα binds strongly to REGβ and weakly to itself; and (3) REGβ binds only to REGα [82]. Furthermore, after being mixed with REGα, REGβ subunits chromatograph as heptamers, not monomers [82]. Thus there appears to be little doubt that α and β subunits are present in the same ring. Whether REGα/REGβ hetero-oligomers are hexamers is very doubtful. First, a variety of physical measurements, including X-ray crystallography, have shown that the REGα oligomer is a heptamer [91,92]. Secondly, various combinations of mutant REGα monomers and REGβ subunits produce hetero-oligomers in which the apparent ratio of β/α subunits is approx. 1.3 as determined by HPLC analysis; a similar value was obtained upon analysis of red-blood-cell 11 S REG [93]. A ratio of 1.3 for REGβ to REGα subunits is consistent with the hetero-oligomer being a heptamer that contains three α and four β subunits. It would, therefore, be surprising to find that the α/β hetero-oligomer is a hexamer. In fact, MS measurements demonstrate that recombinant REGα/REGβ hetero-oligomers contain seven subunits [93]. Thus it seems very likely that natural 11 S REG molecules will prove to be heptamers.

Crystal structure of REGα

A crystal structure of the REGα heptamer has been solved at 2.8 Å resolution [92]. The individual REGα subunits are composed largely of α-helices. Four long helices containing 33–45 residues pack against one another to form the core of the subunit. At the base of each subunit there is a loop that connects helix 2 with helix 3. This loop has been shown to be critical for proteasome activation (Figure 3). A total of 39 residues, Pro^{64}–Gly^{102}, are disordered in the crystal and form a 'loop' on the upper surface of each subunit. This unstructured stretch of amino acids encompasses the homologue-specific KEKE motif of REGα. The last nine amino acids in each REGα subunit are also disordered in the crystal structure. The seven REGα subunits form a barrel-shaped structure measuring 60 Å in height and 90 Å at its widest. A central aqueous channel traverses the barrel. It has a 20 Å diameter opening on one end and a 30 Å opening on the other end, which is presumed to be the proteasome-binding surface. The central channel is lined by charged residues; four lysine residues, four glutamic acid residues and an aspartic acid residue from helix 3 form rings of positive and negative charge on the inner surface of the channel through the heptamer. Virtually all of the remaining residues are polar, so the channel is well suited for permitting the entry or egress of small, water-soluble peptides.

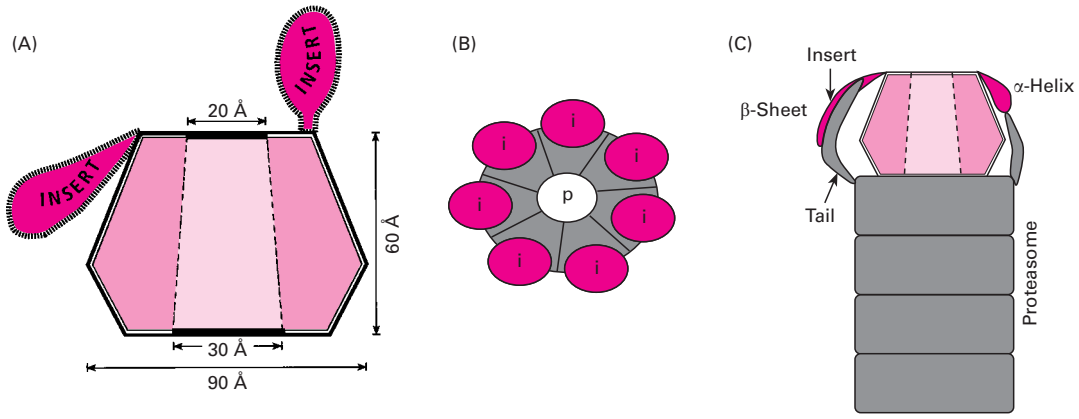

Figure 4 Possible conformation of homologue-specific inserts

(**B**) Schematic representation of the REGα heptamer viewed from the top. The central circle ('p') is the pore leading down to the proteasome, and it is about 20 Å in diameter. Each of the seven peripheral red circles (i) represents the sphere that would be formed if the 39 amino acids (Pro^{64}–Gly^{102}) disordered in the REGα crystal structure adopted a globular conformation. (**A**) Schematic representation of a section of the REGα heptamer as viewed from the side. The dimensions are taken from the crystal structure. Note that only two of the seven inserts (red) are shown, and their shapes are arbitrarily drawn because they are disordered in the crystal structure. (**C**) Potential interactions between REGα inserts (red) and C-terminal tails of proteasome α subunits (grey).

FUNCTIONAL REGIONS WITHIN REG SUBUNITS

Homologue-specific inserts

The homologue-specific inserts are not resolved in the X-ray structure of REGα, presumably because they are flexible. One can, nonetheless, place limits on their positions in the assembled oligomers. As illustrated in Figure 4(A), the inserts could extend as far as 50 Å from the upper surface of the heptamer, depending upon their degree of condensation. They are also arranged on the upper surface of the heptamer close enough to interact easily with each other (Figure 4B). In fact, the REG inserts could, in principle, interact with the C-terminal extensions present on some of the proteasome α subunits (Figure 4C). In this way, the inserts could contribute binding energy for REG proteasome association. Or by binding some proteasome α tails and not others, they might differentially activate proteasome β subunits.

Two recent papers describe the properties of REG molecules from which inserts have been deleted. Song et al. [84] deleted the 28-amino-acid insert from REGα and found no effect on proteasome activation. They did, however, observe impaired hetero-oligomer formation. In a more extensive study, the homologue-specific inserts were deleted (Δi) from all three REG homologues [86]. Both REG$\alpha\Delta$i and REG$\gamma\Delta$i formed heptamers and activated human red-cell proteasomes to the same extent as their full-length counterparts. By contrast, REG$\beta\Delta$i exhibited, at low protein concentrations, reduced proteasome activation when compared with the wild-type REGβ protein. REG$\beta\Delta$i formed hetero-oligomers with REG$\alpha\Delta$i, and the hetero-oligomers, at low concentrations, stimulated the proteasome less than wild-type REGα/REGβ oligomers. These studies demonstrate that the REGα and REGγ inserts play virtually no role in oligomerization or in proteasome activation. Removal of the REGβ insert, on the other hand, reduced binding of this subunit and REGα–REGβ oligomers to proteasomes. The pattern of activated peptide hydrolysis was identical for full-length and Δi versions of each REG homologue. Thus the inserts do not explain why REGα and REGβ activate cleavage of many different fluorogenic peptides, whereas REGγ preferentially activates cleavage of peptides with basic residues next to the fluorescent leaving group.

Activation loops

A recent study that combined PCR mutagenesis with an *in vitro* activity assay resulted in the isolation of 36 inactive, single-site REGα mutants [85]. Most of the mutant proteins were monomers that formed fully active hetero-oligomers when mixed with REGβ. Eight REGα mutants, however, produced partially active REGα–REGβ complexes. Five of these mutants were clustered between Arg^{141} and Gly^{149}; the other three involved mutation of Pro^{240}. As mentioned above, Arg^{141}–Gly^{149} forms a loop at the base of each α subunit, and Pro^{240} contacts this loop directly [92]. Thus random mutagenesis identified a small area on the surface of REGα critical for proteasome activation. One mutation in this loop [N146Y ($Asn^{146} \rightarrow$ tyrosine)] resulted in a REGα heptamer that binds the proteasome tightly but does not activate peptide hydrolysis. Corresponding amino acid substitutions in REGβ (N135Y) and REGγ (N151Y) produced inactive proteins that also bind the proteasome and inhibit proteasome activation by their normal counterparts. Thus REG binding to the proteasome can be separated from activation of the enzyme. It is not known whether REGαN146Y heptamers are inactive because they fail to open a channel into the proteasome or because they do not induce conformational changes in the enzyme's catalytically active β subunits.

C-terminal regions

Several studies indicate that the last ten residues in REG homologues are important for proteasome activation. Whereas the 11 S REG is stable in red-blood-cell lysates, it can be rapidly inactivated in extracts from liver, muscle or kidney [94]. Inactivation was traced to lysosomal carboxypeptidase B, and subsequent treatment of the 11 S REG with yeast carboxypeptidase Y or pancreatic carboxypeptidase B produced a molecule unable to bind proteasomes [94]. Site-directed mutagenesis approaches have confirmed the importance of the C-terminus in proteasome activation. Deletion of REGα's C-terminal tyrosine residue or its conversion into charged amino acids produced heptamers unable to bind the proteasome [84]. Deletional analyses on REGβ produced similar results in that REGβ subunits lacking one, two or nine C-terminal amino acids

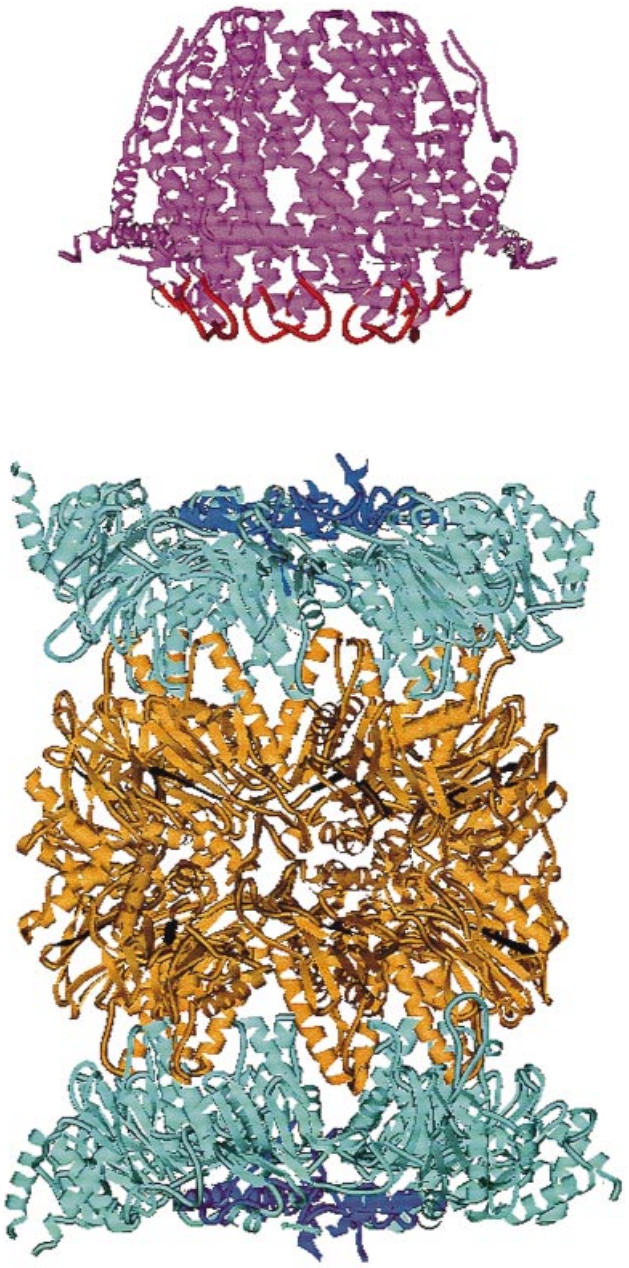

Figure 5 Model for the interaction of REG with the proteasome

Seven-fold axes are aligned for REGα (top; purple) and the 20 S proteasome (below; α- and β-subunits are shown in pale blue and light brown respectively). The activation loop is shown in red. Segments of the proteasome that are most likely to contact REG are in darker blue (residues 13–34) of the α subunits. Reprinted with permission from Nature [92] © 1997 Macmillan Magazines Limited.

did not bind the proteasome [95]. On the assumption that the last ten residues in each REG homologue play an important role in proteasome binding, Zhang et al. exchanged REGα's C-terminal eight residues for those in REGγ, which binds the proteasome tighter than REGα; they found that the REGα241γ8 chimaera bound much tighter than wild-type REGα [95]. However, proteasome activation by REGα241γ8 was identical with that shown by REGα, implying that the C-terminal residues in REG homologues do not determine which proteasome β subunits become activated. More extensive analysis of C-terminal chimaeras between all three REG homologues confirm the importance of REG C-terminal regions in binding the proteasome and further indicate that these regions do not directly participate in the activation of specific proteasome β subunits (J. Li, X. Gao, L. Joss and M. Rechsteiner, unpublished work).

Model for proteasome activation

The N-terminal sequences in α subunits completely seal off the yeast proteasome's internal chambers. With this in mind, it seems likely that binding of REG must cause a conformation change in proteasome α-subunits such as to promote substrate access to, or product release from, the enzyme's active sites. A reasonable model for activation involves the activation loops, Arg^{141}–Gly^{149}, and the last ten residues, Pro^{240}–Tyr^{249}, of REGα subunits. It is notable that, although the last nine amino acids of REGα [residues 241–(Tyr)249] are not resolved in the crystals [92], Pro^{240} is clearly visible and directly touches the activation loop (see Figure 3). These two regions of REGα subunit are therefore well positioned to associate with the N-terminal helices in proteasome α-subunits. If these helices were pulled up and away by the activation loop and the last ten residues of REG, a continuous channel would lead from the upper surface of REGα to the interior of the proteasome (see Figure 5). This alone, however, does not seem sufficient to explain selective increases in hydrolysis of specific fluorogenic peptides [52,53,76]. For this, one must also imagine that association with REG causes a conformational change that is propagated to the catalytically active β-subunits in the proteasome. The crystal structure of REG–proteasome complexes should prove invaluable in determining the mechanism of activation.

Biological properties of REG subunits

For the most part, knowledge of the biological properties of REG lags behind structural information on the molecule. Nonetheless, it seems safe to say that the biological properties of REGα and REGβ are consistent with these two proteins playing a role in Class I immune presentation. For example, all known subunits of the 19 S regulatory complex, except one, are expressed by yeast and humans [16]. But yeasts do not contain genes encoding REG subunits. This indicates that REG does not participate in a fundamental cellular process, but rather serves a biological function confined to higher eukaryotes. More direct indications that REGα and REGβ function in the immune system are provided by their response to immune cytokines and by their relatively high concentration in organs of the immune system.

IFNγ induction of REGα and REGβ

As mentioned above, the cDNA for human REGα was first identified because synthesis of the protein is induced by IFNγ in human keratinocytes [79]. A number of studies have since confirmed that REG mRNAs and proteins are induced by IFN treatment. Ahn et al. [80] treated human renal carcinoma cells with IFNγ and observed persistent increases in REGα and REGβ messages and a transient increase in REGγ mRNA. Very similar results were obtained upon IFNγ treatment of mouse hepatoma cells [96]. Using a transgenic mouse model for IFNγ production in liver cells, Tanahashi et al. [97] reported that IFNγ induces REGα and REGβ mRNAs, but they found that the message for REGγ remained unchanged. Taken together, these three studies demonstrate that IFNγ strongly induces

Table 1 Organ distribution of REG mRNAs (a) and proteins (b)

The abundance of REG proteins or mRNA is expressed as a relative scale from − to + + +. The scoring was performed by M.R., who tried to be consistent when extracting information from the various papers cited. Abbreviations: nd, not done; Ref., reference; UW, unpublished work.

(a) mRNA abundance

Organ ...	Brain	Testis	Heart	Muscle	Kidney	Liver	Lung	Thymus	Spleen	Ref.
REGα										
Mouse	−	+	+ +	+	+/−	+ +	+ +	+ + +	+ +	[92]
Mouse	−	nd	nd	nd	+	+ +	+ +	+	+/−	[94]
Human	+/−	nd	+ + +	+ + +	+	+ +/−	+ +/−	nd	nd	[93]
REGβ										
Mouse	−	+	+	+/−	+/−	+	+ +	+ + +	+ + +	[92]
Mouse	−	nd	nd	nd	+ +	+ +	+ +	+	+/−	[94]
Human	+/−	nd	+ + +	+ + +	+	+ +/−	+ +	nd	nd	[93]
REGγ										
Human	−	nd	+ +	+ +	+/−	+	+ +/−	nd	nd	[93]

(b) Protein abundance

Organ ...	Brain	Muscle	Kidney	Liver	Lung	Thymus	Spleen	Ref.
REGα								
Mouse	+/−	nd	+	+ +	+	+ + +/−	+ + +	[94]
Mouse	+/−	+/−	+	+ +	+ +	+ +	+ + +	J. Li, UW
Rat	+/−	+	+ +	+ +	nd	nd	nd	[89]
REGβ								
Mouse	−	nd	+/−	+	+	+ + +	+ + +	94
Mouse	−	+/−	+/−	+	+	+ +	+ +	J. Li, UW
REGγ								
Mouse	+ +	+/−	+/−	+/−	+/−	+/−	+/−	J. Li, UW

REGα and REGβ mRNAs and only modestly induces the REGγ message.

Realini et al. [78] observed a 5-fold increase in the rate of REGα synthesis following treatment of HeLa cells with IFNγ. Likewise, synthesis of REGα and REGβ was induced by IFNα and IFNγ in HeLa, Raji and Jurkat cells [90]. Tanahashi et al. [97] employed a Western-blotting protocol and found that IFNγ treatment of human SW620 cells produced marked increases in REGα and REGβ proteins; by contrast REGγ actually disappeared! Jun Li in our laboratory also used Western blotting to quantify REG levels, but he obtained different results. Exposure of mouse LK^B cells or human HeLa cells to IFNγ increased the cellular concentration of REGα and REGβ by 3–6-fold, whereas REGγ levels were unaffected in both cell lines (J. Li and M. Rechsteiner, unpublished work). In general, measurements of REG synthesis or protein concentrations are consistent with the majority of reported changes in mRNAs. That is, IFNγ induces REGα and REGβ with little effect on REGγ.

Organ distribution of REGs

Four groups have examined the distribution of REG mRNAs and REG subunits among various organs. DeMartino and his colleagues found that REGα/β protein levels are high in rat kidney and liver, moderate in muscle and very low in brain [94]. Subsequent surveys using mouse organs have produced results consistent with these early findings. Levels of REGα and REGβ are highest in spleen, thymus and lung, moderately abundant in liver and almost absent in brain (see Table 1). However, two reports on the distribution of REGα and REGβ mRNAs disagree substantially. Jiang and Monaco [96] report high levels of REGα and REGβ mRNAs in lung, thymus and spleen, in agreement with higher expression of the two REG subunits in these organs. By contrast, Soza et al. [98] report low amounts of these mRNAs in spleen and thymus. Although the basis for these disparate results concerning REG mRNAs levels is unknown, it seems reasonable to conclude that REGα and REGβ proteins are abundant in immune organs, e.g., spleen, thymus and lung, and almost non-existent in brain. The virtual absence of REGα and REGβ in brain matches the extremely low levels of MHC Class I molecules in nervous tissues. The paucity of REG and MHC Class I molecules in brain can be rationalized by assuming that CTL-mediated destruction of neurons would prove lethal to an organism.

Intracellular distribution of REGs

Ahn et al. [90] used immunofluorescence microscopy and fractionation procedures to determine the intracellular distribution of REGα and REGβ. They reported REGα and REGβ to be present in both nucleus and cytoplasm. Subcellular fractionation demonstrated that some REGα/β molecules were present in the microsomal fraction, but most were found in the cytosol. Similar studies by Soza et al. [98] confirmed that REGα and REGβ are present in the nucleus and in cytoplasm fractions. REGγ was found to be largely nuclear. Their immunocytological analyses produced a surprising result. REGβ was reported to be highly abundant in nucleoli and REGα to be absent from this organelle. Because REGα and REGβ preferentially form hetero-oligomers [81], this is an unusual finding. It may be incorrect, since Wilk and his colleagues did not observe such an intranuclear distribution of REGα and REGβ subunits in HeLa or NT2 neuronal precursor cells [99]. Rather, they found REGα and REGβ to be mostly cytoplasmic, with nucleolar staining seen for both subunits. REGγ was found to be largely nuclear, but absent from the nucleolus. In addition, anti-REGγ antibody labelled two

Table 2 Summary of REG properties

	REGα	REGβ	REGγ	REGα/REGβ
Subunit molecular mass	28589 Da	27 230 Da	29 365 Da	–
pI	5.87	5.44	5.78	–
Oligomeric state	Heptamer	Monomer	Heptamer	Heptamer
Affinity for proteasome	++	+/−	+++	++++
Activation properites	↑ Cleavage after acidic basic hydrophobic residues	↑ Cleavage after acidic basic hydrophobic residues	↑ Cleavage after basic residues	↑ Cleavage after acidic, basic, hydrophobic residues
IFNγ	↑	↑	0 or ↓	↑
Ca^{2+} binding	+	+	+	Not known
Cellular location	Cytoplasm and nucleus	Cytoplasm and nucleus	Predominantly nuclear	Cytoplasm and nucleus
Evolutionary distribution	Vertebrates	Vertebrates	Vertebrates, insects and worms	–
Tissue distribution	Widespread; highest in immune organs	Widespread; highest in immune organs	Widespread	–

structures in the cytoplasm. One consists of microtubular-like extensions dispersed by nocodazole. The other structures are most likely autophagosomes. In view of the markedly different REG distributions seen in mouse fibroblasts [98] and HeLa cells [99], additional localization studies are clearly needed.

Chromosomal location of REG genes

IFNγ induces the synthesis of TAPs, the proteasome subunits LMP2 and LMP7 and MHC Class I molecules themselves [100]. These components in the Class I presentation pathway are encoded by genes located in the MHC complex. Whether the genes for REGα and REGβ also reside in either MHC locus is of obvious interest. Several recent papers report that they are not. Kandil et al. [101] mapped the tightly linked REGα and REGβ mouse genes close to Atp5g1 locus on chromosome 14; mouse REGγ mapped close to the Brca1 on chromosome 11. A second gene encoding mouse REGβ has been mapped to a LINE1 element [102]. McCusker et al. [103] mapped the human REGα and REGβ genes to human chromosome band 14q11.2 and more recently characterized the chromosomal region around the REGα and REGβ loci [104]. The human MHC complex is on chromosome 6 and the mouse MHC complex is on chromosome 17. Thus, unlike the peptide transporters (TAPs) and IFNγ-inducible proteasome LMP subunits, the genes for REGs are not present in the MHC region.

Additional properties of REG

REGα and REGβ are reasonably long-lived proteins. Ahn et al. [90] measured their metabolic stability in cells and reported half-lives of 30 h for both proteins. Each of the three REG homologues binds calcium. It was found that calcium binding inhibits peptide hydrolysis by REGα–proteasome complexes [105], but recent studies indicate that this effect is only seen at calcium concentrations above 200 μM [82]. Because the levels of cytosolic Ca^{2+} are in the low-micromolar range, the effect of calcium on REG–proteasome activity is of questionable physiological significance. It has also been reported that the 11 S REG must be phosphorylated in order to activate the proteasome [106]. This is a surprising result, in view of the fact that recombinant REG homologues are active and, according to MS measurements, they are not phosphorylated [82]. For this reason, we do not believe that 11 S REG subunits require phosphorylation for activity. Finally, it has been reported that the potent immunosuppressive drug rapamycin inhibits induction of REGβ expression by phytohaemagglutinin [107]. In Table 2 we have summarized various properties of 11 S REG and each REG subunit.

CLASS I ANTIGEN PRESENTATION

The proteasome as a source of Class I epitopes

There is considerable evidence that proteasomes generate many of the peptides presented on Class I molecules. Lactacystin, a fungal metabolite that inhibits the proteasome with reasonable specificity, markedly reduces Class I antigen presentation [13,14]. Proteasomes have been shown to cleave peptide precursors *in vitro*, generating products structurally similar to Class I epitopes [108,109], and, in several cases, lack of epitope presentation correlates with altered proteasomal cleavage of precursor peptides [110–112]. IFNγ, which induces many components of the Class I pathway, including TAPs and Class I molecules themselves, also induces the synthesis of three proteasome subunits and REGs α and β [12]. Taken together, these findings provide strong circumstantial evidence that the proteasome and 11 S REG play important roles in the production of Class I epitopes. It is, however, by no means clear that the proteasome is involved in the generation of all Class I peptides, since there are several convincing demonstrations that lactacystin-mediated inhibition of the proteasome actually enhances the presentation of a specific Class I epitope [113–115].

Properties of Class I epitopes

The Class I immune response is extremely versatile. Thousands of peptides derived from an organism's own proteins and from viral pathogens are presented by a handful of Class I molecules. Individual humans express, at most, six different MHC Class I molecules, each of which can present hundreds of different peptides. This remarkable plasticity is inherent in the structure of the MHC Class I molecule. Peptides are bound in a cleft or groove between two α-helices. The floor of the cleft is formed by β strands, and the cavity can accommodate a wide range of peptides [116]. However, the ends of the cleft are closed, thereby restricting the length of Class I epitopes. For this reason, virtually all peptides that bind Class I molecules are between eight and eleven amino acids long, with the vast majority being nine or ten residues [117,118]. Although a given MHC Class I molecule can present many different epitopes, the presented peptides share common features. For example, peptides presented by the human MHC Class I molecule HLA-A2 often have leucine at position 2 and branched-chain residues at the C-terminus. By contrast, human HLA-B27 presents peptides with arginine or lysine at the C-terminus, and arginine is frequently found at position 2. Class I epitopes show the greatest restriction at the C-terminus, with almost all Class I epitopes having basic or hydrophobic C-

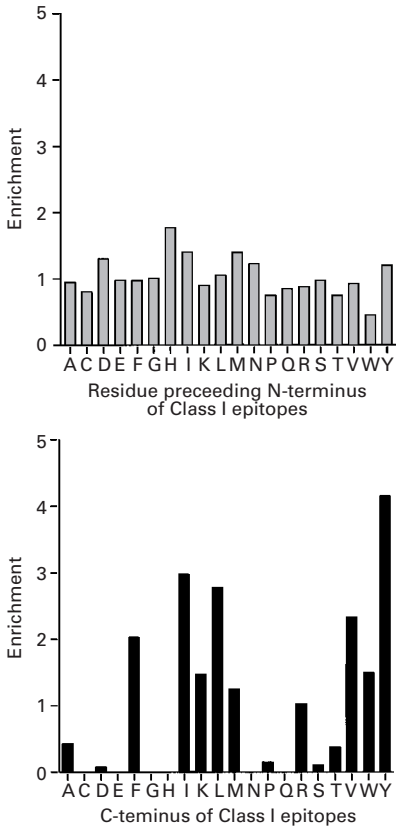

Figure 6 Residues flanking Class I epitopes

We have compiled a library of 338 Class I epitopes for which precursor proteins can be identified with reasonable confidence. The epitopes were analysed with respect to the amino acid residues immediately preceding the epitope and the residue present at the epitope's C-terminus. The relative abundance of a specific amino acid at these positions was divided by its relative abundance in the precursors to yield a numerical estimate of enrichment. As shown in the panel at the top, there is little bias in the residues preceding Class I epitopes, whereas there is marked enrichment for hydrophobic and branched-chain amino acids at the C-terminus of Class I epitopes (bottom panel). Amino acids are shown on the abscissae in one-letter code.

termini; by contrast, there is little residue bias just preceding the N-terminus of Class I epitopes (see Figure 6). Are the peptide binding preferences of Class I molecules governed principally by structural constraints on the Class I proteins themselves? Or do they also reflect the substrate specificity of the proteases that generate the peptides and the specificity of the TAPs that pump Class I epitopes into the ER lumen?

TAP specificity

Microsomes containing functional TAPs transport peptides in an ATP-dependent reaction. Whereas peptides of less than eight amino acids are poor substrates for TAPs, peptides of up to 40 residues can be moved into the ER lumen [119,120]. The optimal length for transport, however, ranges from nine to twelve amino acids. Mouse and human TAPs are reasonably permissive with regard to peptide sequence. For example, human TAP prefers basic and hydrophobic residues at the C-terminus, but will tolerate any residue except proline at that position. There is little restriction at the N-terminus or at internal positions in the peptide. On the other hand, a comparison of mouse, rat and human TAPs by Elliott [119] reveals that, in general, TAPs prefer peptides that terminate in basic or hydrophobic residues. Thus transporter specificity seems to mirror the preferences of MHC Class I molecules for peptides with non-polar or positively charged C-termini.

Proteasome specificity

As mentioned above, there are five activities in the mammalian proteasome: the T-L, CT-L, PGPH, BrAAP and SNAAP. If proteasome specificity matches that of the MHC Class I molecules, one would expect the CT-L, T-L and BrAAP catalytic sites to be more active than the PGPH or SNAAP sites, because most Class I epitopes end in basic or hydrophobic residues. However, as measured with fluorogenic peptides, the CT-L, T-L and PGPH activities are comparable using proteasomes from non-immune tissues or from cells not exposed to IFNγ. It has been shown that, following IFNγ treatment, three catalytically active proteasome subunits, X, Y and Z, are replaced by LMP7, LMP2 and subunit MECL respectively [121–126]. This raises the possibility that INFγ-induced subunits confer distinct catalytic properties upon proteasomes, and with this in mind, a number of groups have compared the substrate specificity of proteasomes containing the IFNγ-induced subunits with proteasomes containing the X, Y and Z subunits. Several early studies reported that the activity of proteasomes containing LMP2 and LMP7 is higher against substrates with hydrophobic or basic residues at the P1 position and lower against acidic substrates [127–131]. Other groups did not observe significantly increased cleavage of the CT substrate, succinyl-Leu-Leu-Val-Tyr 7-(4-methyl)-coumarylamide, and only modest decreases in PGPH activity [128,132]. Two groups even reported decreased CT-like activity [133,134].

The effects of IFNγ-induced proteasome subunits on enzyme specificity have also been assayed using synthetic peptides, with results almost as controversial as those just cited. Boes et al. [133] used a 25-residue peptide containing a Class I epitope from murine cytomegalovirus pp89 as substrate and found that proteasomes from IFNγ-treated cells did not increase the yield of the Class I epitope, but did produce apparent precursors to the epitope. Transfection of LMP2 and/or LMP7 into lymphoblasts lacking genes from these proteasome subunits produced similar results [135]. By contrast, Ehring et al. [132] found that LMP2/LMP7-positive and LMP2/LMP7-negative proteasomes did not differ significantly in their degradation of insulin B chain or a peptide epitope from histone H3.

Why such disparate results have been obtained in the attempts to assess the impact of LMP subunits on proteasome specificity is not clear. In the various studies cited, proteasomes were isolated by different procedures and were of different states of purity when assayed. This may account for the disparate results. Whatever the cause for the widely variable results, the best answers to this question, in our opinion, are provided by the studies of Eleuteri et al. [134]. They used highly purified proteasomes that were convincingly either LMP2-, LMP7- and MECL-positive (bovine spleen) or -negative (bovine pituitary). Unless bovine proteasomes are atypical, we think it is safe to conclude that proteasomes containing LMPs and MECL1 exhibit markedly enhanced BrAAP activity and reduced PGPH and CT-like activities.

Specificity changes induced by the 11 S REG

Since REGα and REGβ subunits are also induced by IFNγ, one cannot focus on the catalytic properties of proteasomes alone. In cells exposed to IFNγ there will likely be elevated levels of REG–proteasome complexes, and these could be the major source of peptides destined for Class I molecules. Small fluoro-

Table 3 Stimulation of proteasome active sites by 11 S REG

Abbreviations: RBC, red blood cells; MCA, 7-(4-methyl)coumarylamide; pNA, *p*-nitroanilide; amino acids are shown using the one-letter notation; s, succinyl. References: [a]Dubiel et al. [53]; [b]Di Cola [75]; [c]Ma et al. [52]; [d]Kuehn and Dahlman [150]; [e]Ustrell et al. [76].

	Stimulation (-fold)				
Active site	Human RBC[a]	Human RBC[b]	Bovine RBC[c]	Rabbit muscle (P)[d]/rabbit RBC (REG)	Human lymphoblast (P)[e]/human RBC (REG)
T-L					
PFR-MCA	10	–	–	–	10
LSTR-MCA	–	10	–	–	–
VLR-MCA	–	–	5	–	–
VGR-MCA	–	–	–	15	–
PGPH					
LLE-pNA	50	60	2	10	15
CT-L					
sLLVY-MCA	50	50	25	15	30

genic peptides have been widely used to assay the effects of REG on multiple catalytic activities exhibited by proteasomes. The results of such studies are summarized in Table 3, where it can be seen that 11 S REG generally stimulates the CT-like and PGPH active sites to a greater extent than the T-L active site. In a study relevant to antigen presentation, Ustrell et al. [76] compared the effect of 11 S REG on proteasomes containing or lacking LMP2 and LMP7 subunits. For the most part, there was little difference in the extent of activation, except for two peptides with hydrophobic C-termini. These two peptides, Gly-Gly-Phe 7-(4-methyl)-coumarylamide and Leu-Tyr 7-(4-methyl)coumarylamide, were cleaved 3- and 6-fold faster respectively by LMP-positive proteasomes. The 11 S REG increased hydrolysis of the two peptides by LMP-positive proteasomes even more, so that the difference became 10-fold for each peptide.

Several groups have used synthetic peptides to assay the effects of REG on cleavage-site preference. Groettrup et al. [136] reported that REG markedly changed both the quality and quantity of peptides produced upon digestion of a 25-residue peptide from murine cytomegalovirus pp89. Subsequently, the same groups reported that proteasomal generation of MHC Class I epitopes was optimized by REG-induced co-ordinated dual cleavages [137]. Shimbara et al. [138] also reported that 11 S REG promotes dual cleavage in two specific peptides, but for the most part, the proteasome activator only speeded cleavages that occurred in its absence. The two peptides for which REG promoted a specific cleavage are characterized by short sequences to one side of the cleavage site. This led the authors to hypothesize that sequences flanking the epitope function as anchors to trap peptides for dual cleavage. Finally, Niedermann et al. [139] also report enhanced production of dual cleavage products by recombinant REGα, but they emphasize that the observed changes were quantitative and not qualitative.

There is little doubt that REG stimulates hydrolysis of synthetic peptides by the proteasome. Consequently, it increases the yield of dual cleavage products. However, it is much less certain that REG induces *co-ordinated* dual cleavages. In fact, there are problems with this idea, because the subunit responsible for the major CT-like activity (subunit X) or BrAAP activity (LMP7) is not adjacent to another active proteasome β subunit (see Figure 1). Therefore, it is virtually impossible for an epitope precursor to span two LMP7 subunits with only eight to ten intervening

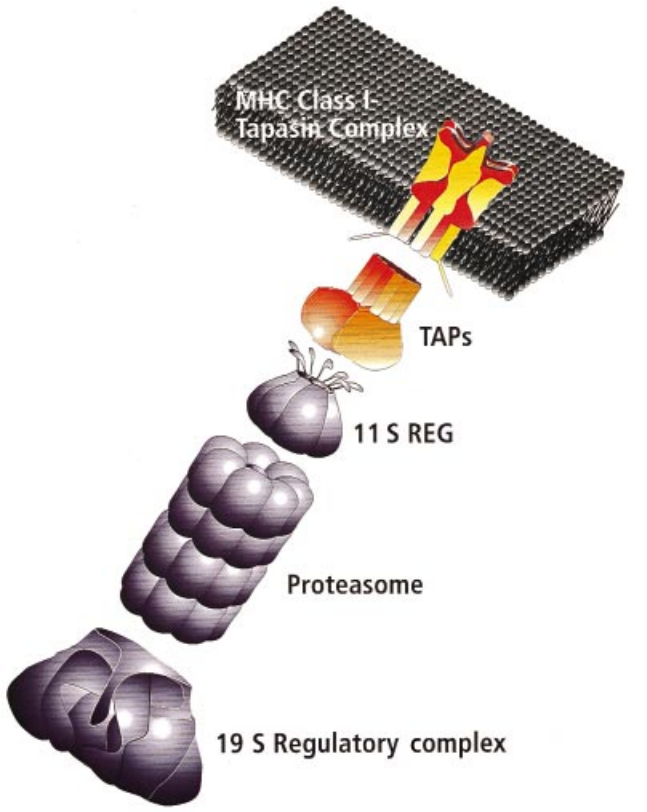

Figure 7 Schematic representation of the idea that REGα/β couples the proteasome to the MHC Class I peptide loading complex

Four MHC Class I and four tapasin molecules are shown embedded in a lipid bilayer. The vase-like TAP1/TAP2 transporter has been pulled out of the membrane for clarity. 11 S REG is shown just below TAP, and the 20 S proteasome is aligned with REG. Finally, the 19 S regulatory complex of the 26 S proteasome is shown at the far lower left. Hendil et al. [17] have presented evidence that the 19 S regulatory complex and 11 S REG can simultaneously bind the proteasome.

amino acids. But, since there is evidence that LMP2 exhibits CT-like activity and not the PGPH activity of subunit Y [140], a precursor could simultaneously bind LMP2 and MECL-1 with only eight to ten amino acids spanning the two active sites, and LMP2 might generate peptides with the hydrophobic C-termini characteristic of Class I epitopes. Still, the problem remains that almost any amino acid can be found preceding the N-termini of Class I epitopes (see Figure 6). If most Class I epitopes are generated by the co-ordinated action of MECL1 and LMP2, then MECL1 must exhibit exceptionally broad specificity. In addition, there is good evidence that N-terminal trimming of epitope precursors can occur in the ER [141,142]. This coupled with the demonstration that lactacystin prevents presentation of a C-terminally extended *ova* epitope, but not N-terminally extended *ova* epitopes [143], strongly suggests that proteasomes generate the C-termini of Class I epitopes. Different enzymes, presumably aminopeptidases, generate their N-termini. It is relevant in this context that leucine aminopeptidase is induced by IFNγ [144].

REGs and the molecular-coupling hypothesis

The ability of REG$\alpha\beta$ to cause the proteasome to generate multiply cleaved fragments from long peptides may fully explain

its role in Class I antigen presentation. On the other hand, REG homologues contain unique inserts that do not contribute to oligomerization, proteasome binding or activation. The inserts are, nonetheless, conserved in evolution, implying that they have a biological function. From their location on the 'upper' surface of REG heptamers, it seems reasonable to suppose that the inserts couple the proteasome to other cellular components. Two sets of molecules are good candidates as upper-surface binding partners for REG$\alpha\beta$. Heat-shock proteins could transfer unfolded proteins through REG$\alpha\beta$ rings into proteasomes for subsequent degradation. Since there are studies implicating heat-shock proteins in antigen presentation [145–148], this idea is consistent with the proposed roles of REGα and REGβ in the immune system. A more attractive hypothesis to us would have REG$\alpha\beta$ heptamers connect the proteasome to TAP–tapasin–MHC I complexes in the ER membrane. The Class I pathway provides one striking example of 'metabolic channelling' in that the TAPs are associated with 'empty' MHC I molecules [149]. Consequently, newly transported peptides are released in the immediate vicinity of their intended carriers. Because there are cytosolic peptidases that might degrade freely diffusing peptides, one can imagine that peptides generated within the proteasome are transferred directly to TAPs (see Figure 7). In this way, the 11 S REG would provide a protective tunnel from proteasome to TAP. As an extension of this hypothesis, we propose that REGγ couples proteasomes to nuclear components, which could include kinetochores, the inner nuclear membrane, etc.

SUMMARY AND PERSPECTIVES

In the 8 years since the discovery of 11 S REG (PA28), we have learned a great deal about the structure of these proteasome activators. Each of three REG homologues, α, β and γ, activates the proteasome. Recombinant REGα and REGγ subunits form heptamers, and REGβ subunits form heteroheptamers with REGα. A crystal structure for REGα has revealed that seven REG subunits form a ring that surrounds an aqueous channel. Mutagenesis studies have identified a highly conserved stretch of nine amino acids critical for activation, and this region forms a loop on the presumed proteasome-binding surface of each subunit. Removal of homologue-specific inserts from REGα or REGγ has no effect on proteasome activation, but deletion of a single C-terminal residue severely impairs REG association with the proteasome. We still do not know how REG activates the proteasome. A major goal for the future is to obtain the crystal structure of the REG–proteasome complex, which should solve this problem.

In contrast with the considerable structural information on REGs, clear insights into their biological function(s) are still lacking. To be sure, there is strong circumstantial evidence that REG α/β heptamers play some role in Class-I-antigen presentation, but it is not known whether they merely speed multiple cleavages in epitope precursors or whether they also channel peptides to TAP–tapasin–MHC I complexes. We obviously need a more detailed picture of the role of REGs in the Class I pathway. Similarly, we want to know what REGγ is doing in the nucleus. Several laboratories are constructing REG knockout mice, and these organisms may clarify the roles of REG$\alpha\beta$ and REGγ.

Perhaps there are two central unanswered questions concerning the REG proteasome activators. Do they merely promote the entry and/or release of small peptides to and from the proteasome? Or do REG molecules also couple the proteasome to other cellular structures? If the latter is the case, then two-hybrid screens or direct-binding assays should identify REG-binding components other than the proteasome. If the former is true, such approaches are destined for failure. Despite the risks, attempts to find proteins that bind the upper surface of REG heptamers are underway. Hopefully, these attempts will provide the much-needed information on the biological function(s) of REG$\alpha\beta$ and REGγ.

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