Characterization of Recombinant REGα, REGβ, and REGγ Proteasome Activators*

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Full-length cDNAs for three human proteasome activator subunits, called REGα, REGβ, and REGγ, have been expressed in Escherichia coli, and the purified recombinant proteins have been characterized. Recombinant α or γ subunits form heptameric species; recombinant β subunits are found largely as monomers or small multimers. Each recombinant REG stimulates cleavage of fluorogenic peptides by human red cell proteasomes. The pattern of activated peptide hydrolysis is virtually identical for REGα and REGβ. These two subunits, alone or in combination, stimulate cleavage after basic, acidic, and most hydrophobic residues in many peptides. Recombinant α and β subunits bind each other with high affinity, and the REGβ/REGb heteromeric complex activates hydrolysis of LLVY-methylcoumaryl-7-amide (LLVYMCA) and LLE-β-nitroanilide (LLE-βNA) more than REGα or REGβ alone. Using filter binding and gel filtration assays, recombinant REGγ subunits were shown to bind themselves but not α or β subunits. REGγ differs from REGα and REGβ in that it markedly stimulates hydrolysis of peptides with basic residues in the P1 position but only modestly activates cleavage of LLVY-MCA or LLE-βNA by the proteasome. REGγ binds the proteasome with higher affinity than REGα or REGβ yet with lower affinity than complexes containing both REGα and REGβ. In summary, each of the three REG homologs is a proteasome activator with unique biochemical properties.

The proteasome is a large multisubunit protease found in eukaryotes, prokaryotes, and archaeabacteria (1, 2). The enzyme from the archaeabacterium Thermoplasma acidophilum has served as an important structural model for proteasomes from higher organisms. It is composed of two kinds of subunits called α and β (3). These two proteins associate to form four rings stacked upon one another to produce a cylinder roughly 10 nm in diameter and 15 nm in length (4–6). Seven α subunits form each of the end rings with two rings of seven β subunits sandwiched between them (7, 8). The recently determined x-ray structures of the archaeabacterial and yeast enzymes (9, 10) have shown that the two internal β-rings generate a buried chamber containing 14 active sites approximately 30 Å apart from each other. In higher eukaryotes, there are 7 genes encoding α subunits and 10 genes encoding β subunits (11). The β subunits confer upon the proteasome at least five distinct activities that cleave peptide bonds following basic, acidic, hydrophobic, branched chain, and small neutral amino acids (12, 13). In higher eukaryotes, the two α-rings interact with regulatory complexes to produce either a large 26 S energy-dependent protease or an activated peptidase. A 700-kDa regulatory complex composed of approximately 15 subunits binds eukaryotic proteasomes to form the 26 S protease that degrades ubiquitylated or unmodified proteins in an ATP-dependent fashion (14, 15). In the absence of ATP, an 11 S protein complex can bind to either one or both ends of the proteasome and stimulate its peptidase activities up to 60-fold. This oligomeric complex, known as the PA28 or 11 S REG (16, 17), is composed of two highly conserved and homologous subunits that we called REGα and REGβ (18, 19).

The proteasome, either as part of the 26 S protease or as an activated peptidase, has been implicated in a variety of cellular processes. These include cell cycle progression (20, 21), selective proteolysis (15, 22, 23), and antigen presentation on class I major histocompatibility (MHC-1) molecules (for reviews, see Refs. 2, 24, and 25). With regard to antigen presentation, the proteasome has been shown to cleave peptide precursors in vitro, generating products with structural properties similar to MHC-I epitopes (26–28). This proteolytic capacity is enhanced by REGα (18); LMP2 and LMP7 (29, 30), purified human REG (16); or a combination of LMPs and REG (31, 32). These in vitro findings suggest that the proteasome and its regulators play an important role in the processing of antigenic peptides. In support of this idea, in vivo evidence indicates that LMP2 and LMP7, two proteasome β subunits, are up-regulated by γ interferon (33). A third proteasome β subunit, MECL1 (34), and the α, β, and γ subunits of the 11 S REG (18, 19) as well as other components of the antigen presentation pathway such as TAP1, TAP2, and MHC-I molecules are also induced by IFNγ (35). Furthermore, mice lacking genes for LMP2 or LMP7 exhibit reduced capacity to present antigens on MHC-I molecules (36, 37). And recently, it has been reported that overexpression of REGα subunits in mouse cells leads to increased antigen presentation (38). Taken together, these findings constitute strong circumstantial evidence that the 11 S REG and the proteasome are involved in production of peptides presented on MHC-I molecules.

We proposed earlier that the Ki antigen, a protein homologous to the REGα and REGβ subunits, would also be a proteasome activator (39). In this study, we have characterized the activation properties of recombinant REGα, REGβ, and Ki.

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1 The abbreviations used are: MHC-I, class I major histocompatibility complex; βNA, β-naphthylamide; Boc, t-butyloxycarbonyl; IPTG, isopropyl-β-D-thiogalactopyranoside; MCA, methylcoumaryl-7-amide; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase HPLC; Suc, succinyl; Ub, ubiquitin; PBS, phosphate-buffered saline.
antigen, which we call REG. We have found that although REGα and REGβ subunits can independently activate the peptidase activity of the proteasome, together these two proteins form a superactivating αβ complex. The γ subunit did not interact with either α or β subunits. REG is, nonetheless, able to bind the proteasome and activate hydrolysis of certain fluorogenic peptides.

EXPERIMENTAL PROCEDURES

Materials—The fluorogenic peptides Boe-Phe-Ser-Arg-MCA (FSR-MCA), Boe-Leu-Val-Lys-MCA (VLK-MCA), Boe-Glu-Arg-MCA (GRK-MCA), N-glut-Gly-Gly-MCA (GGF-MCA), Suc-Leu-Leu-Val-Tyr-MCA (LLV-MCA), Suc-Ala-Ala-Phe-MCA (AAP-MCA), Boe-Leu-Arg-MCA (LRR-MCA) and Pro-Arg-MCA (PFR-MCA) were obtained from Peninsula Laboratories Inc. (Belmont, CA). Z-Gly-Gly-Leu-MA (GGL) was from Bachem Biosciences Inc. (King of Prussia, PA), while benzyloxycarbonyl-Leu-Leu-Glu-β-MNA (LEL-MNA), Suc-Leu-Tyr-MCA (LYM-MCA) and ubiquitin were from Sigma. Nitrilotriacetic acid membrane filters (0.1 μm) were from Schleicher and Schuell. Affinity-purified peroxidase-conjugated, goat anti-rabbit IgG was from Organon Teknika (West Chester, PA). The Renaissance Western blot chemiluminescence reagent was obtained from DuPont NEN. The Sequenase® DNA-sequencing kit was from U.S. Biochemical Corp., and the Gene Amp® polymerase chain reaction (PCR) components were purchased from Perkin-Elmer Cetus. Sequencing grade (α-332P)ATP (1000 Ci/μmol), t-(35S)Methionine (>80 Ci/μmol), [α-32P]CTP, and [γ-32P]ATP were supplied by NEN Life Science Products, and amino acid mixtures were obtained from Promega (Madison, WI). The λ ZAP II DNA library, the pBluescript KS, and the XL1 Blue Escherichia coli strain were all obtained from Stratagene (San Diego, CA), while the BL21 (DE3) E. coli strain was from Novagen (Madison, WI), and the Library Efficiency DH5α competent cells were from Life Technologies, Inc. Reverse transcriptase avian myeloblastosis virus, NeI, ClaI, SphI, isopropyl-β-thiogalactosidase (IPTG), and hog brain calmodulin were from Boehringer Mannheim.

Isothermal and Characterization of DNA Clones—The cloning and in vivo expression of the α subunit of human regulator was described earlier (18). During the screening for REGs, a 235-bp partial DNA for REGα was isolated. Using this partial clone, α-32P-labeled random-primed probes were generated (Random Primed DNA Labeling Kit, Boehringer Mannheim) and used to screen approximately 108 bacterial clones from the λ ZAP II HeLa cell DNA library as described for the cloning of REGα (18). A full-length clone for REGβ activator was isolated, sequenced, and subcloned into the pAED4 expression vector. The published sequence of Kα-antigen (40) was used as a nondegenerate oligonucleotide primer specific for the 3′-untranslated region of the REGγ gene (5′-AGACCGACATGGCC-3′). The primer served to generate a gene-specific DNA upon reverse transcription of CaCl₂-purified total HeLa cell RNA with reverse transcriptase avian myeloblastosis virus. The resulting cDNA and two primers specific for the N and C terminus of the REGγ gene (5′-ATGGCCCTCGTTCG-3′ and 5′-AGAGACGCTCACG-3′, respectively), were used in a PCR. The EdiSeg, MegAlign, and Protein algorithms (DNASTAR Inc., Madison, WI) and data base were used to analyze the nucleotide and deduced amino acid sequences of cloned PCR products. Mutation-free clones were subcloned into expression vectors as described below.

Expression and Purification of Recombinant REGα, REGβ, and REGγ Proteins—Recombinant REGα was prepared as described previously (18). Full-length cDNAs for REGα and REGβ were ligated into the NdeI/ClaI or NdeI/BamHI sites, respectively, of the T7 polymerase-dependent expression vector pAED4. Recombinant E. coli BL21(DE3), treated with IPTG according to the manufacturer’s instructions, were lysed in 10 mM Tris, pH 8.8, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, and 1.0 mM dithiothreitol. The cell lysate was centrifuged at 39,000 x g for 30 min at 4 °C. The soluble recombinant proteins were purified by ion exchange chromatography on a DEAE Sephadex A50 column (Pharmacia Biotech Inc.) using a 0–1 M KCl gradient in TDS, pH 8.8, followed by sizing chromatography on a Superdex 200 (26/60) column equilibrated in 10 mM Tris, pH 8.8, 200 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, and 1.0 mM dithiothreitol. The purity of the recombinant proteins was determined by Coomassie Blue and silver staining of gels and the presence of contaminants resolved on SDS-polyacrylamide gels. The identity and integrity of the recombinant proteins were assessed by Edman degradation and electrospray mass spectrometry. The purified recombinant proteins were tested for the presence of contaminating proteases and peptidases using ubiquitylated or unmodified 125I-lysyl-lysyl-lysyl-lysine and a panel of 12 fluorogenic peptides. In particular, the preparations were tested for EcoRI ATP-dependent and HaIVU protease activity using GGL-MCA, AAF-MCA, and LLVY-MCA as substrates (41).

By a combination of PCR and cloning approaches, we have isolated cDNAs for a number of REG variants. Four of these REG mutants were expressed in E. coli and purified. The four mutant REGs are REGWT, which contains a 17-amino acid extension at the C terminus and forms an inactive monomer; REGGm, an inactive nonmonomer; and REGmα, an inactive heptamer. These four proteins served to control for the presence of E. coli factors that might copurify with the recombinant REGs and fortuitously stimulate the proteasome or cleave fluorogenic peptides directly. In all cases, the purified mutant REGs were completely inactive as proteasome activators. Only those purified fractions did cleave any fluorogenic peptides. We therefore conclude that none of the activities reported in this paper can be attributed to E. coli proteins.

Production of Homolog-specific Antibodies and Dot Blot Analysis of Recombinant REGα and REGβ—Recombinant proteins purified from BL21(DE3) E. coli were applied to a nitrocellulose filter using a dot blot apparatus. The filter was stained with Ponceau S, blocked for 60 min in 5% nonfat milk in TBS-T (25 mM Tris, pH 7.5, 0.9% NaCl, 0.04% Tween 20), and then incubated with polyclonal rabbit serum (1:2000 dilution) directed against either REGα or REGβ, washed in TBS, and then incubated for 4 h in the presence of peroxidase-conjugated goat anti-rabbit IgG. The peroxidase reaction was performed at room temperature following the manufacturer’s instructions for the Renaissance Western blot chemiluminescence reagent and exposed to X-Omat AR film.

ELECTROPHORESIS—Denaturing SDS-polyacrylamide gels were composed of 4% stacking gel (10% Tris-HCl, pH 6.8, 1%, SDS) and a 10–20% resolving gel (37.5 mM Tris, pH 8.6, 200 mM glycine, and 0.1% SDS) with a Mini-Protein apparatus (Bio-Rad). Following electrophoresis, proteins were visualized by staining in 0.2% Coomassie Brilliant Blue in 22.5% methanol and 7.5% acetic acid or by silver staining (Bio-Rad).

Spectrofluorometric Proteasome Assays—Spectrofluorometric assays were performed in the presence of 100 μM fluorogenic peptides and various amounts of proteasome and REGα, REGβ, or REGγ in a final volume of 100 μl of 10 mM Tris, pH 7.45. Proteasome and recombinant REG proteins were incubated for 10 min at 25 °C prior to the addition of fluorogenic peptide substrates. Reactions were performed at 25 °C or at temperatures indicated in the figure legends and were terminated by adding 200 μl of cold 100% ethanol. Fluorescence was measured with a Perkin-Elmer fluorometer using excitation at 380 nm and emission at 440 nm for peptides containing MCA; hydrolysis of substrates containing βNA was monitored at excitation and emission wavelengths of 335 and 410 nm, respectively. Proteasomes were purified from human red blood cells as described previously (44).

125I-Lysyl-lysyl-Ubiquitin Conjugate Proteolysis—Activated Xenopus egg extracts were prepared as described, and 125I-lysyl-lysyl-Ub conjugates were prepared as described (Refs. 45 and 46, respectively). Xenopus egg extracts (22.5 μg) were mixed with 25 μl of 125I-lysyl-lysyl-Ub conjugates (approximately 420 cpn/μl) or unmodified 125I-lysyl-lysyl (approximately 1000 cpn/μl) and various amounts of REGα, REGβ, and REGγ. Alternatively, purified human proteasomes or proteasome-REG complexes were tested for the presence of ATP-dependent or ATP-independent proteolytic activity using conjugated or unmodified 125I-lysyl-lysyl. The incubations were at 23 °C, and the fraction of hydrolysis of 125I-lysyl-lysyl was determined by acid precipitation (45).
FIG. 1. Properties of three REG homologs. A, aligned primary sequences of human REGα (18), REGβ, and REGγ (40). Amino acid sequences were deduced from the nucleotide sequences using the MegAlign algorithm (Clustal method). The insert of each REG is shown black on white. A potential protein kinase C recognition site shared by REGα and REGβ is boxed. Differences from the published sequence of the REGβ are circled; Ser21 and Asn229 were previously identified as Ala2 and Thr229, respectively (19). B, physicochemical properties of REGα, REGβ, and REGγ. cMr, calculated molecular masses (kDa) for the recombinant REGs lacking Met1; eMr, experimental masses for the recombinant REGs determined by electrospray mass spectrometry; pI, calculated isoelectric points; nMr, the estimated native molecular masses (kDa) were determined by sizing chromatography (SC) as described under “Experimental Procedures” and dynamic light scattering (DLS) using a DynaPro-801 TC instrument (ProteinSolutions, Charlottesville, VA). The theoretical data and sequence similarity values were obtained using the ProteinPilot algorithm. The experimental values were obtained as described under “Experimental Procedures.” C, Coomassie Blue R250 staining of recombinant human REGs separated by SDS-polyacrylamide gel electrophoresis. S, molecular mass standards (kDa).

NaCl, 1.1 mM MgCl2, 0.1 mM EDTA, and 10 mM dithiothreitol. The elution of REGα, REGβ, REGγ, REGmβ, and REGmκ was compared with the elution positions of calibration proteins II (Combitek, Boehringer Mannheim). Selected fractions (20 μl) were analyzed for proteasome-stimulating activity using LLVY-MCA or LKR-MCA as substrates. Proteins in active fractions were separated by RP-HPLC as indicated in the figure legends. Alternatively, selected fractions were transferred onto a nitrocellulose filter after separation on a denaturing SDS-polyacrylamide gel or directly applied to the filter using a dot blot apparatus. The fractions were then analyzed for the presence of α, β, or γ subunits using homolog-specific antibodies.

Cloning, Expression, and Puriﬁcation of Recombinant Human REGα, REGβ, and REGγ—cDNAs encoding the α and β subunits of the human 11 S REG were isolated from a ZAPII cDNA library containing HeLa sequences. A cDNA for REGγ was ampliﬁed from puriﬁed HeLa cell RNA using PCR primers designed on the basis of the published sequence (40). The deduced amino acid sequences for the α and β subunits match those published earlier (18, 40). The nucleotide and deduced amino acid sequences of the HeLa REGβ subunit agree with the published sequence except for Ser92 and Asn229, which were identiﬁed by Ahn et al. (19) as Ala2 and Thr229, respectively. Interestingly, position 229 is also Asn in the REG homologs.

RESULTS

Cloning, Expression, and Purification of Recombinant Human REGα, REGβ, and REGγ—cDNAs encoding the α and β subunits of the human 11 S REG were isolated from a ZAPII cDNA library containing HeLa sequences. A cDNA for REGγ was ampliﬁed from puriﬁed HeLa cell RNA using PCR primers designed on the basis of the published sequence (40). The deduced amino acid sequences for α and β subunits match those published earlier (18, 40). The nucleotide and deduced amino acid sequences of the HeLa REGβ subunit agree with the published sequence except for Ser92 and Asn229, which were identiﬁed by Ahn et al. (19) as Ala2 and Thr229, respectively. Interestingly, position 229 is also Asn in the REGβ subunit from rats (19). Fig. 1A presents the sequence of each subunit aligned to emphasize the homology between the proteins except for highlighted regions that we call subunit-specific “inserts.” The degree of sequence similarity between the three REG homologs is 35–50% using the J. Hein or Clustal methods (Fig. 1B).

Expression of each REG subunit in E. coli BL21(DE3) was initiated by the addition of IPTG to growing cells, and the recombinant proteins were puriﬁed by ion exchange chroma-tography, followed by gel ﬁltration (see “Experimental Procedures”). Each subunit constituted more than 90% of the protein pooled after gel ﬁltration (see Fig. 1C). The molecular masses determined by electrospray mass spectrometry (Fig. 1B) are in
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Enzymatic reactions consisted of 100 μl of 10 mM Tris, pH 7.45, containing 340 ng of human red blood cell proteasomes and 6 μg of REGα, REGβ, and REGγ. Proteasomes and REGs were incubated for 10 min at 25 °C prior to addition of fluorogenic peptides to a final concentration of 100 μM. The reactions were stopped after 15–40 min by addition of 200 μl of ethanol. The markedly reduced activation of LLVY-MCA and LLE-pNA hydrolysis by REGγ cannot be attributed to cleavage of alternate bonds within these two fluorogenic peptides, since addition of aminopeptidase did not increase fluorescence. In fact, aminopeptidase addition did not produce increased fluorescence for any combination of substrates and enzymatic components presented in the table. This indicates that the amino acid-MCA and amino acid pNA bonds are the exclusive sites of hydrolysis. The values (fold stimulation, S) were calculated as follows: S = Fprot + REG/Fprot, where Fprot and Fprot+REG are the fluorescence values resulting from the cleavage of fluorogenic substrates with proteasomes and proteasome-REG complexes, respectively. Each value represents an average of four to six measurements.

Table I
Substrate specific stimulation of the proteasome by REGα, REGβ, and REGγ

| Peptide | REGα | REGβ | REGγ |
|---------|------|------|------|
| Basic   | 26   | 18   |      |
| PFR     | 14   | 10   |      |
| GKR     | 13   | 10   |      |
| LRR     | 35   | 20   |      |
| Acidic  |      |      |      |
| LLE     | 12   | 4    |      |
| Hydrophobic |      |      |      |
| LLVY    | 54   | 5    |      |
| AAF     | 21   | 11   |      |
| GGF     | 2    | 1    |      |
| LYY     | 17   | 12   |      |

* Johnston, S. C., Whitby, F., Reallini, C., Rechsteiner, M., and Hill, C. P. (1997) *Protein Sci.*, in press.

Good agreement with masses expected for each protein lacking its N-terminal methionine. Size exclusion chromatography demonstrated a marked difference in the apparent size of REGβ as compared with REGα and REGγ. The latter two proteins chromatographed with apparent molecular weights of about 200,000. By contrast, REGβ eluted from Superdex 200 at a size expected for a monomer or dimer (see Fig. 6). Extensive structural analysis, including equilibrium sedimentation and x-ray crystallography, demonstrate that recombinant REGα is a heptamer.2

Proteasome Activation by REGα, REGβ, and REGγ—Each recombinant REG subunit was tested for its ability to stimulate peptide hydrolysis by proteasomes. Human red blood cell proteasomes were incubated with increasing amounts of each recombinant subunit, and hydrolysis of LLVY-MCA was measured. It is clear from the activation profiles shown in Fig. 2A that the three subunits differ in their ability to stimulate cleavage of LLVY-MCA. Although high concentrations of REGα and REGβ produce equivalent extents of activation, the two homologs differ significantly at lower concentration. Activation by REGγ is biphase, with modest stimulation of LLVY-MCA cleavage at concentrations below 30 ng/μl and stimulation equivalent to REGα at higher concentrations (Fig. 2B).

Because the individual REG subunits diverge significantly at the insert region (see Fig. 1), we suspected that they might differentially activate cleavage of specific fluorogenic peptides. For this reason, we assayed the ability of each homolog to stimulate hydrolysis of 10 fluorogenic peptides. The results presented in Table I show that REGα and REGβ are very similar in their ability to activate hydrolysis of specific peptides by the proteasome. They markedly activate hydrolysis of fluorogenic peptides containing basic, acidic, or hydrophobic amino acids in the P1 position. By contrast, REGγ stimulates cleavage of fluorogenic peptides with positive residues adjacent to the fluorescent leaving group. It is particularly striking that REGγ barely activates cleavage of LLVY-MCA or LLE-pNA, two substrates that are highly responsive to REGα and REGβ.

Effects of REG Homologs on Km and Vmax—Further comparison among the three activators is provided by kinetic studies performed with three peptides frequently used as model substrates: LRR-MCA, LLE-pNA, and LLVY-MCA. Increasing concentrations of each substrate were incubated with proteasomes or proteasome-activator complexes. The graphs in Fig. 3 show that REGα complexes exhibit higher activity than equimolar amounts of either REGβ or REGγ. Again, it was found that REGγ preferentially stimulates the cleavage of...
stimulation of the proteasome by REGa were not greatly affected by the REG homologs, Vb. While the K$m$ values increased 20–40-fold.

FIG. 3. Activity of proteasome-REG complexes as a function of substrate concentration. Proteasomes (425 ng) and increasing concentrations of LLE, LLVY, or LRR were incubated for 10 min at 24 °C in the absence (20 S) or in the presence of REG homologs (9 μg of REGα, -β, -γ, or -αβ), as indicated in the panels. The fluorescence resulting from the hydrolysis of the substrates was determined as described under “Experimental Procedures.” Plots show the initial velocity (nmol of peptide cleaved per minute per mg of proteasome) as a function of the substrate concentration.

The chromatographic behavior of mixtures of recombinant REG subunits provided further evidence for strong associations between α and β subunits. Purified REGβ subunits were mixed with equal amounts of either REGα or REGγ and chromatographed on Superdex 200 fractogel. Comparison of the elution

FIG. 4. Temperature-dependent stimulation by recombinant REGs. The hydrolysis of LLVY (100 μM) with human red cell proteasomes was determined at incubation temperatures between 16 and 46 °C in the absence (20 S) or in the presence of saturating amounts of either REGα (α) or REGβ (β). The hydrolysis of LRR-MCA by proteasomes (20 S) and proteasome-REGγ complexes (γ) is shown in the inset. The fluorescence resulting from the hydrolysis of the substrates was determined as described under “Experimental Procedures.”
profiles in Fig. 6B with that in Fig. 6C show that β subunits elute as heptamers or hexamers after being mixed with REGα.

Western blot analysis, RP-HPLC separation, and Edman degradation revealed that the hexa-/heptameric species eluting at 160 ml in Fig. 6C contained equal amounts of both α and β subunits. In agreement with the data shown in Figs. 2 and 3, the newly formed αβ heteromer exhibited approximately 2-fold higher proteasome-stimulating activity than the same amount of the REGα homomer (Fig. 6D). By itself REGβ did not stimulate the proteasome, since the concentrations present in the tested fractions were below the threshold required for activation (see Fig. 2B). In contrast, REGγ oligomers did not bind REGβ (Fig. 6, E–G). Electrophoretic analysis of selected fractions from the profiles shown in Fig. 6, E–G, failed to reveal major changes in the elution of either REGβ or REGγ (not shown). Furthermore, the REGγ activity profile was virtually unchanged after mixing with REGβ (Fig. 6H).

The possibilities that individual β subunits bind to the surface of α heptamers and are simply carried along during gel filtration or that REGα heptamers promote assembly of REGβ homo-oligomers were excluded using a mutant REGα subunit. A number of inactive REGα proteins have been generated by PCR mutagenesis. Many do not form heptamers by themselves, but they readily become active multimers when mixed with REGβ subunits. An example of the conversion of an inactive monomeric REGα mutant (REGmo) and wild type REGβ monomers to a higher molecular weight, active multimer is shown in the gel filtration profiles in Fig. 7, A–C. Both REGmo and REGβ migrate as monomers (Fig. 7, A and B, respectively), but the REGmo/REGβ mixture eluted as an oligomer (Fig. 7C). Neither monomeric species by itself activated the proteasome, but the mixed heteromer was active (Fig. 7D). Selected fractions from the profiles shown in Fig. 7, A–C, were analyzed by denaturing SDS electrophoresis (Fig. 7, E–G). The stained gels show that, when fractionated individually, REGmo and REGβ chromatograph as monomers (solid arrowheads in panels E and F, respectively), but when mixed they elute at a position corresponding to heptamers or hexamers. Although REGmo and REGβ subunits did not resolve in the gel shown in Fig. 7G, both subunits were present at an apparent 1:1 molar ratio upon RP-HPLC (Fig. 7H). We consider the formation of active oligomers from two inactive monomeric proteins, REGβ and REGmo, as very strong evidence that the two proteins form a heteromeric complex.

Association of REGα, -β, and -γ with the Proteasome—The
fact that REGα, -β, or -γ stimulates peptide hydrolysis demonstrates that each is capable of binding the proteasome. Two experiments indicate that the individual REG subunits differ in their affinity for the enzyme. The first experiment exploits the different activation properties of REGα, REGβ, and REGγ. As shown in Fig. 2, REGγ stimulates the proteasome to hydrolyze LRR-MCA to a greater extent than LLVY-MCA. REGα or REGβ, on the other hand, stimulates cleavage of the two peptides almost equally. These properties allowed us to perform competition experiments. Proteasomes were mixed with saturating amounts of REGα or REGβ, and increasing amounts of REGγ were added. As shown in Fig. 8, high levels of REGγ suppress hydrolysis of LLVY-MCA activated by REGα (closed squares) or REGβ (open diamonds). We interpret the observed inhibition as a result of REGα or REGβ being displaced from the proteasome by the added REGγ oligomers. Inhibition was not observed when proteasomes activated by a mixture of REGα and REGβ were challenged with increasing amounts of REGγ (Fig. 8, closed circles). Thus, the competition experiments indicate that REG species bind the proteasome with decreasing affinities in the order α/β > γ > α or β.

Further support for differential proteasome binding by REG homologs was obtained using a direct binding assay. In this assay, REG homologs competed for binding to antibody-tethered proteasomes, and the identities of the proteasome-bound REGs were determined using homolog-specific antibodies. Results from the competition experiments are presented in Fig. 9, where the competing species are indicated for each dot blot. It is evident that REGγ displaced REGα from the proteasome but did not compete away REGαβ complexes (Fig. 9, upper panel), and as expected from the enzymatic assay in Fig. 8, only REGαβ heteromeric complexes markedly reduced REGγ binding to the proteasome (Fig. 9, lower panel). Thus, both enzymatic and direct binding assays demonstrate that REGαβ binds the proteasome tighter than REGγ, which in turn binds tighter than REGα or REGβ.

REGα, -β, and -γ Bind Ca2+—We have previously shown that REGα and a Ub fusion protein containing the REGα KEKE insert (Ub-KEKE) are able to bind Ca2+ (51). Using a 46Ca2+ overlay assay, we compared the calcium binding properties of the three REG homologs, calmodulin, ubiquitin, and Ub-KEKE. REGα, -β, and -γ, the Ub-KEKE fusion protein, and calmodulin bind 46Ca2+ in the presence of 5 mM MgCl2, whereas no signal could be detected for ubiquitin (Fig. 10). We also showed previously that the addition of calcium inhibited peptide hydrolysis by REGα-proteasome complexes (51). When we repeated these measurements, concentrations of Ca2+ that were 3–4-fold higher than previously reported were needed to inhibit peptide cleavage by proteasome-REGα complexes (not shown). The reason for this discrepancy with our previous findings is not known. DNA sequencing indicates that no mutations occurred in our expression plasmid, so we suspect that the difference reflects some unexplained variability in our proteasome and/or recombinant REG preparations. However, the results in Fig. 10 and those in Ref. 51 do show that each of the recombinant REGs can bind calcium, although with a lower affinity than other well characterized Ca2+-binding proteins such as calmodulin.

The 26 S Proteasome Is Unaffected by High Levels of REG Homologs—It has been shown that REGs do not promote
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Each recombinant REG. Purified human proteasomes were unable to degrade 125I-lysozyme or 125I-Ub-lysozyme conjugates in the presence of REGα, REGβ, REGγ, or REGαβ complexes (data not shown). Furthermore, amounts of recombinant REG homologs (molar concentrations estimated to be 2000-fold higher than the 26 S proteasome) were unable to prevent hydrolysis of 125I-Ub-lysozyme conjugates in Xenopus egg extracts (not shown). These results confirm that the 11 S REGs or individual REG subunits do not convert the proteasome to an enzyme capable of degrading 125I-lysozyme or 125I-Ub-lysozyme conjugates. Furthermore, they do not impair the function of the 26 S proteasome in Xenopus laevis egg extracts.

**DISCUSSION**

The experiments presented in Figs. 3 and 4 demonstrate that each of the three REG homologs, α, β, and γ, activates peptide hydrolysis by the proteasome. For REGα and REGγ, this observation is not surprising, since the two proteins are closely related (Fig. 1), each forms a defined oligomer of about 200 kDa (Fig. 6), and recombinant REGα was shown previously to activate the proteasome (18). The finding that REGβ is a proteasome activator might be considered unexpected for two reasons. First, there are two reports that REGβ does not activate the proteasome (2, 53). Second, we have produced 31 monomeric mutants of REGα that cannot stimulate peptide hydrolysis by the proteasome.10 Because REGβ chromatographs on Superdex 200 as a monomer (see Fig. 6), one might expect it to be inactive as well. However, relatively high concentrations of REGβ are required to detect its activity. In fact, REGβ appears to be inactive in Figs. 7 and 8 because the fractions were assayed prior to concentration. High concentration might promote REGβ heptamers or hexamers that are either required for or are more efficient at proteasome activation. Alternatively, monomers of REGβ may assemble into a heptameric ring only on the proteasome surface. It should be noted that dynamic monomer-oligomer equilibrium for β subunits may explain the biphasic activation curve obtained for this REG homolog (see Fig. 2B).

The activity of REGβ is difficult to detect for another reason: it is temperature-sensitive. Although the data in Fig. 4 show REGβ active at 42 °C, other REGβ preparations were markedly inactivated at 35 °C. Accordingly, enzyme reactions incubated at 37 °C may not detect REGβ activity, and this is the reason that we assayed the recombinant REG homologs at room temperature rather than at 37 °C as in previous studies (18, 53). In conclusion, we are confident that REGβ activates the proteasome, but its activity is more difficult to detect than that of REGα or REGγ.

Although each REG homolog activates the proteasome, there are differences in the extent to which they stimulate hydrolysis of specific fluorogenic peptides. REGα and REGβ activated the cleavage of 9 of the 10 peptides listed in Table 1. Indeed, the two proteins produce virtually identical patterns of stimulated peptide hydrolysis. By contrast, REGγ barely increased the cleavage of LLE-βNA and LLVY-MCA, yet it activated the proteasome to hydrolyze all of the peptides with Arg or Lys next to the fluorescent leaving group. As noted in the Introduction, REGs have been implicated in the generation of peptides presented to the immune system on MHC-I molecules. Present peptides are 8–11 residues long, and their C termini almost always consist of hydrophobic or basic amino acids (54). In this context, it appears that of the three REG homologs, γ would produce peptides best suited for class I presentation, since it does not markedly increase hydrolysis after the negatively charged residue in LLE-βNA. Of course, this inference rests on the controversial assumption that the substrate specificities obtained using small fluorogenic peptides reflect cleavage preferences in

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**Fig. 9. Direct binding assay for relative association of REG homologs with proteasomes.** Analysis of proteasome-bound REGs. Specific antibodies directed against REGα (upper panels) or REGγ (lower panels) were used to detect REG binding to proteasomes in a competition assay. REG homologs were incubated with antibody-tethered proteins and rinsed, and the bound species were identified using homolog-specific antibodies (see details under “Experimental Procedures”). Upper panel, REGα (α) or a 1:1 (w/w) REGα/REGβ mixture (αβ) was incubated with equimolar amounts (γ), or 2-fold excess (γγ) of REGγ. Lower panel, REGγ (γ) competed with equimolar amounts of REGα (α), REGβ (β), or a REGαβ mixture (αβ). The specificity of the antibodies is illustrated in the panels on the right where REGα and REGγ were directly blotted on the membrane and probed with anti-α (upper panel) or anti-γ (lower panel) antibodies.

**Fig. 10. Binding of 45Ca2+ to recombinant REG homologs.** 1 and 5 μg of purified recombinant REGs (α, β, γ); REGα/REGβ (αβ); α, a ubiquitin fusion protein extended at the C terminus with the KEKE insert of REGα (DPVKKEKEKEEKKQKEKDDKEKEKKGEDEDDK (Ub-KEKE)); and calmodulin (CaM) were applied to a nitrocellulose filter using a slot blot apparatus. The filter was exposed to 45Ca2+ (2 μCi/ml) in 10 mM imidazole, pH 6.8, 60 mM KCl, 5 mM MgCl2, at room temperature for 15 min (64), briefly rinsed with distilled water, and exposed to x-ray film.

hydrolysis of any fluorogenic peptide that is not already a substrate of the proteasome (32), and they do not convert the proteasome to an enzyme capable of degrading intact, folded proteins (16, 17). It is known that the red blood cell 11 S REG is displaced from proteasomes by the regulatory complex of the 26 S proteasome (52). We have reexamined these points using
longer, naturally occurring peptides and proteins (see Refs. 32 and 55 for discussions on this point).

The distinct patterns of enhanced peptide hydrolysis produced by the REG homologs provide clues regarding the mechanism(s) by which they activate the proteasome. The recently published x-ray structure of the yeast proteasome reveals that the enzyme's active sites are virtually inaccessible from the particle's surface (10). Hence, it seems almost certain that activators like the 19 S regulatory complex (44, 56, 57) or 11 S REGs (16, 17) increase substrate access to the central proteolytic chamber within the proteasome. The generation of channels leading into the proteasome could be the only mechanism for activation of peptide hydrolysis. For example, association of the rabbit 19 S regulatory complex with the proteasome results in a uniform 3-fold faster hydrolysis of LLE-\(\beta\)NA, PFR-MCA, and LLVY-MCA (52). Since it is well established that each of these peptides is hydrolyzed by a specific proteasome \(\beta\) subunit (58–60), equivalent stimulation for each fluorogenic peptide can be interpreted in favor of increased substrate access or product egress as the mechanism by which the 19 S regulatory complex activates peptide hydrolysis. Alternatively, binding of proteasome activators might also induce conformational changes that increase the catalytic efficiency of one or more proteasome \(\beta\) subunits. This would be consistent with activation by the 11 S REGs, since they increase the hydrolysis of specific peptides to widely varying extents. Furthermore, as shown in Table I, the pattern of activation by REG\(\gamma\) differs significantly from that produced by REG\(\alpha\) or REG\(\beta\). Taken at face value, the data in Table I indicate that all three REG homologs activate the proteasome trypsin-like active site(s), whereas only REG\(\alpha\) and REG\(\beta\) activate the subunit(s) largely responsible for cleavage of LLE-\(\beta\)NA and LLVY-MCA. We conclude that REG homologs activate proteasomes both by opening channels to the particle's central chamber and by directly activating specific subunits in the proteasome \(\beta\)-rings.

Comparisons presented in Fig. 1B show that the amino acid sequences of REG\(\gamma\) and REG\(\gamma\) diverge about equally from the sequence of REG\(\alpha\). With this in mind, it is interesting that REG\(\alpha\) and REG\(\beta\) activate hydrolysis of specific peptides in an identical manner quite distinct from the pattern of activation produced by REG\(\gamma\). The insert regions of REG\(\alpha\) and REG\(\beta\) may explain their equivalent activation properties at saturation. Although the REG\(\beta\) insert is shorter than that of REG\(\alpha\), both inserts are highly charged regions characterized by “alternating” lysine and glutamate residues. We have previously speculated that such regions, known as KEKE motifs, promote association of proteasomes with the 19 S regulatory complex and the 11 S regulator (61). Here, we suggest that interaction between the KEKE motifs found at the C termini of proteasome \(\alpha\) subunits, C6 and C9, and the KEKE inserts in REG\(\alpha\) and REG\(\beta\) results in enzymatic activation of those proteasome \(\beta\) subunits responsible for cleaving LLE-\(\beta\)NA and LLVY-MCA. The distinctly different sequence of amino acids in REG\(\gamma\)'s insert would, according to this model, explain its inability to stimulate hydrolysis of these two fluorogenic peptides. Alternatively, the inserts may promote homolog-specific association or bind other cellular factors. The ideas that REG inserts influence peptide cleavage specificity or partner selection are, without doubt, speculative. Fortunately, these ideas can be readily tested by reciprocal transfer of insert regions between REG homologs.

Several experiments have revealed highly specific interactions among the REG homologs. The gel filtration profiles in Fig. 6 demonstrate strong interactions between REG\(\alpha\) and REG\(\beta\) subunits and the absence of interaction between REG\(\gamma\) and REG\(\beta\). The conversion of REG\(\beta\) monomers to a distinct oligomeric species when mixed with REG\(\alpha\) heptamers (Fig. 6, B and C) can be rationalized by assuming that the REG\(\alpha\) heptamers are in equilibrium with a small pool of \(\alpha\) monomers. The addition of \(\beta\) subunits would shift the equilibrium to a mixed oligomer because of the high affinity between \(\alpha\) and \(\beta\) subunits. The absence of interaction between REG\(\alpha\) and REG\(\gamma\) could result either from a lack of inherent affinity between the two proteins or from the absence of a significant monomer-heptamer equilibrium for REG\(\gamma\). The filter binding assay in Fig. 5 favors the first possibility, since REG\(\gamma\) subunits clearly bind themselves but not REG\(\alpha\) or REG\(\beta\). Presumably, the assay in Fig. 5 works because filter-bound monomers or small oligomers of each REG are in conformations that permit formation of specific subunit interfaces with their binding partners. Thus, both solution experiments and filter binding assays indicate that among REG homologs the principal oligomeric species will consist of \(\alpha\)/\(\beta\), \(\alpha\)/\(\gamma\), and \(\gamma\)/\(\gamma\) subunits.

The absence of oligomers of recombinant REG\(\beta\) and its high affinity for \(\alpha\) subunits strongly suggest that REG\(\beta\) subunits will be present as hetero-oligomers with REG\(\alpha\) in the 11 S complexes isolated directly from mammalian cells. Recent data also indicate that REG\(\alpha\) and REG\(\beta\) are coordinately regulated by \(\gamma\)-interferon in a fashion distinct from the regulation of REG\(\gamma\) (19). These findings taken together suggest that 11 S REGs obtained from mammalian cells will likely consist of distinct REG\(\gamma\) heptamers or REG\(\alpha\)-REG\(\beta\) complexes. Whether these presumed REG\(\alpha\)-REG\(\beta\) oligomers will be found to contain six or seven subunits remains, in our opinion, an open question.

The recombinant REG homologs not only display distinct affinities for each other; they bind the proteasome with characteristic affinities. The enzymatic competition assays in Fig. 8 and the direct binding assays in Fig. 9 yield a consistent hierarchy of proteasome binding in which heteromeric REG\(\alpha\)/REG\(\beta\) complexes bind tighter than REG\(\gamma\) and these two complexes bind tighter than either REG\(\alpha\) or REG\(\beta\) alone. There is no reason to believe that these measurements are in error. Still, they may not be physiologically relevant for two reasons. First, it seems likely that proteasomes are in excess within mammalian cells (62). Consequently, there may not be significant competition in vivo. Second, isoelectric variants of both REG\(\alpha\)s and REG\(\beta\)s are present in the 11 S REG isolated from human red blood cells (17). Quite possibly, \(\alpha\) and \(\beta\) subunits are posttranslationally modified in mammalian cells, and such modifications could affect their affinities for proteasomes and/or for each other.

In summary, we have shown that each of three recombinant REG homologs is a proteasome activator with distinct biochemical properties. Previous comparisons between recombinant REG\(\alpha\)s and the 11 S REG purified from red blood cells revealed little difference in their ability to activate peptide hydrolysis by the proteasome (32). Presumably, the properties reported here will apply to the 11 S REG species isolated directly from mammalian cells. But this is clearly a presumption in view of potential modifications to the REG homologs synthesized in mammalian cells. Characterization of REGs purified from mammalian tissues, especially REG\(\gamma\), and comparison with the recombinant REG species is under way. This approach should provide further insight into the roles of the proteasome activators in vivo.

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