# Proteasome Activator 11S REG or PA28: Recombinant REGα/REG$\beta$ Hetero-oligomers Are Heptamers[1]

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ABSTRACT: The proteasome activator 11S REG or PA28 is a conical molecule composed of two homologous subunits, REGα and REG$\beta$. Recombinant REGα forms a heptamer, whereas recombinant REG$\beta$ is a monomer. When mixed with REG$\beta$, a monomeric REGα mutant (N50Y) forms an active hetero-oligomer in which the molar ratio of REG$\beta$ to REGα(N50Y) is close to 1.3. This apparent stoichiometry is consistent with the REGα(N50Y)/REG$\beta$ hetero-oligomer being a heptamer composed of three α and four $\beta$ subunits. Chemical cross-linking of the α/$\beta$ oligomers revealed the presence of REGα−REG$\beta$ and REG$\beta$−REG$\beta$ dimers, but REGα−REGα dimers were not detected. The mass of the REGα(N50Y)/REG$\beta$ hetero-oligomer determined by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) is 194 871 ± 40 Da in good agreement with the theoretical mass of 194 856 Da for an α3$\beta$4 heptamer. Hexamers were not observed in the mass spectrum. For wild-type REG subunits coexpressed in bacteria cells at an apparent $\beta$/α molar ratio of ∼1.2, the resulting hetero-oligomers observed by ESI-TOF MS were again predominantly α3$\beta$4 heptamers, with trace amounts of α4$\beta$3 heptamers also present. On the other hand, the mass spectrum contained a mixture of α7, α6$\beta$1, α5$\beta$2, and α4$\beta$3 heptamers when the REG$\beta$/REGα ratio was 0.1. Thus, formation of heptamers is an intrinsic property of recombinant REGα and REG$\beta$ subunits. On the basis of these results, we propose that 11S REG purified directly from eukaryotic cells is also heptameric, likely α3$\beta$4 or a mixture of α3$\beta$4 and α4$\beta$3 species.

The proteasome, first described by Wilk and Orlowski in bovine pituitary extracts (*1*), is found in bacteria, archaea, and eukaryotes (*2*). Recent structural studies on the proteasome from the archaebacterium *Thermoplasma acidophilum* and the yeast *Saccharomyces cerevisiae* indicate that the structure of proteasomes from all species is quite similar (*3*, *4*). Proteasomes are barrel-shaped molecules assembled from four stacked rings. In the *Thermoplasma* proteasome, each end ring consists of seven identical α subunits, whereas 14 identical $\beta$ subunits form the two inner rings. In eukaryotes, the two α rings are composed of seven distinct α subunits, and each of the two inner rings also contains seven different $\beta$ subunits.

The 19S regulatory complex and 11S regulator (REG)[1] are protein complexes that bind the end rings of the proteasome and activate its peptidase activities (*5*). The 19S regulatory complex consists of 18 distinct subunits, and it associates with the proteasome in an ATP-dependent reaction to form the 26S proteasome. This large energy-dependent protease not only removes abnormal proteins, it also controls a variety of important cellular processes such as cell cycle progression and gene expression by degrading important regulatory proteins (*2*, *5*, *6*−*8*). The 11S REG is a molecule composed of two homologous subunits, REGα and REG$\beta$, that are reported to form a six- or seven-membered ring (*9*−*12*). Its association with the proteasome dramatically activates the proteasome to hydrolyze fluorogenic peptide substrates in vitro, although 11S REG does not promote the degradation of intact proteins (*13*, *14*).

The 11S REG is being extensively studied since it appears to play an important role in Class I antigen presentation (*15*, *16*), and it provides an excellent opportunity to understand how the proteasome can be activated. For instance, it has been shown that recombinant REGα and REG$\beta$ subunits can, by themselves, activate the proteasome to a very similar extent (*11*). However, when mixed, the two subunits preferentially form hetero-oligomers that activate the proteasome to a greater extent than either subunit alone (*11*). Solution of the REGα crystal structure demonstrates that recombinant REGα forms a heptamer (*17*). A highly conserved stretch of 10 amino acids in REGα and REG$\beta$ is critical for proteasome activation (*18*), and this region forms a loop on the presumed proteasome binding surface of each REGα subunit in the crystallized heptamer (*17*). Removal of the 25−30 residue, homolog-specific inserts from REGα

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[1] DEAE, diethylaminoethyl; DTT, dithiothreitol; ESI-TOF MS, Electrospray ionization time-of-flight mass spectrometry; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; REG, 11S proteasome activator; SDS, sodium dodecyl sulfate.

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or REG$\gamma$ subunits, has no effect on oligomerization or proteasome activation (*19*). But deletion of one or a few amino acids from the C-terminus of REGα (*12*, *20*) and REG$\beta$ (*21*) abolishes proteasome binding, and it is known from the crystal structure that the C-terminal regions are adjacent to the activation loops (*17*). Finally, it has recently been shown that both α and $\beta$ subunits contribute equally to proteasome activation by recombinant REGα/REG$\beta$ hetero-oligomers (*21*).

Despite these advances in understanding the structural and functional properties of recombinant REG molecules, it is still not clear whether the α/$\beta$ hetero-oligomer is a hexamer or a heptamer. Song et al. (*22*) proposed that 11S REG or PA28 is a α3$\beta$3 hexamer based on the identical patterns of cross-linked products from 11S REG after staining with either anti-REGα or anti-REG$\beta$ sera. Ahn et al. (*23*) also arrived at a hexameric model based on an apparent REGα to REG$\beta$ ratio of 1.0 in [$^{35}$S]methionine-labeled 11S REG complexes. However, recombinant REGα clearly forms a heptamer (*17*). In addition, if 11S REG were a hexamer, there would be symmetry mismatch upon its association with the proteasome. Because the existence of REGα heptamers and the apparent symmetry mismatch pose problems for the hexamer model, we have employed three techniques to reexamine the number of subunits present in REG hetero-oligomers. By measuring the stoichiometry of REGα to REG$\beta$ both in recombinant and natural hetero-oligomers, by using chemical cross-linking reagents and by measuring the molecular mass of recombinant α/$\beta$ hetero-oligomers with electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS), we conclude that formation of heptamers, not hexamers, is an intrinsic property of REGα and REG$\beta$ subunits. In light of these findings, we propose that natural 11S REG or PA28 molecules are also heptamers.

## EXPERIMENTAL PROCEDURES

*Formation and Purification of Recombinant REGα(N50Y)/REGβ Hetero-oligomers*. The expression and purification of REGα(N50Y), REG$\beta$, and various mutant proteins used in this study were performed as described (*11*, *18*, *21*). After REGα(N50Y) and REG$\beta$ subunits were purified to near homogeneity, equal amounts of the two subunits were mixed and incubated at 4 °C overnight. The resulting mixture was then applied to a Superdex 200 column, and fractions containing REGα/REG$\beta$ hetero-oligomers were used for the analysis of REG$\beta$ to REGα ratios.

*Coexpression of REGα and REGβ in Escherichia coli*. Wild-type REGα cDNA in the plasmid pET11a (with an ampicillin-resistance gene) and wild-type REG$\beta$ cDNA in pET26b (with a kanamycin-resistance gene) were mixed together at a ratio of 1:1 or 1:4 and transformed into BL-21(DE3) by electroporation. Transformants were then selected on Amp/Kan plates. Ten colonies were pooled, inoculated into 1 L of LB culture medium, and grown at 30 °C. At an $A_{600}$ of 0.05, they were diluted into 4 L of LB. When the $A_{600}$ reached 0.2, the bacteria were induced with 0.4 mM IPTG for 2 h. Recombinant hetero-oligomers were purified as described (*18*). To overexpress REGα relative to REG$\beta$, a pAED4 plasmid encoding REG$\beta$ was transformed into BL-21(DE3). Electro-competent cells were then prepared following a standard protocol (*24*) and transformed with pET26b containing the REGα coding sequence. The expression and purification of hetero-oligomers containing REGα and REG$\beta$ was then performed as described above. By this procedure, the apparent molar ratios of REG$\beta$ to REGα ranged from 0.1 at the front of the gel filtration peak to 0.15 in the back fractions.

*Proteasome Activation Assays*. Proteasome activation by REGα/REG$\beta$ hetero-oligomers in 10 mM Tris, pH 7.5, was performed as described (*11*, *18*). To determine whether REGα/REG$\beta$ hetero-oligomers activate the proteasome in 10 mM ammonium bicarbonate, pH 7.5, hetero-oligomers in 0.5× TSD (TSD = 10 mM Tris pH 7.0, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, and 1.0 mM DTT) were first concentrated to about 100 $\mu$L using a Centricon 10 filter. Then 2 mL of cold 10 mM $NH_4HCO_3$ buffer were added to the Centricon 10, which was then centrifuged at 5000*g* at 4 °C for 1 h for buffer exchange. This process was performed five additional times, and the REGα/REG$\beta$ hetero-oligomers were used for proteasome activation assays immediately in 10 mM $NH_4CO_3$, pH 7.5.

*Determination of Subunit Ratios in REGα/REGβ Hetero-oligomers by HPLC*. These experiments were performed as described (*19*). Briefly, fractions from a Superdex 200 gel filtration column were applied to a Vydac 218PP54 C18 column 4.6 mm × 250 mm, and a gradient of 30 to 70% acetonitrile in 0.1% trifluoroacetic acid was used to separate REGα from REG$\beta$. The ratio of REG$\beta$ to REGα was calculated by the following formula: (HPLC peak area of REG$\beta$/theoretical mass of REG$\beta$)/(HPLC peak area of REGα/theoretical mass of REGα). The theoretical masses used were calculated by Editseq program of DNASTAR, and the first methionine was not included in the mass calculation. REGα and REG$\beta$ peak areas were computed by an HP Chemstation program (version A.0 4.01, Hewlett-Packard) based on the absorbance at 214 nm.

*Chemical Cross-Linking and Detection of Dimers*. REGα/REG$\beta$ hetero-oligomers in 0.5× TSD were dialyzed extensively against 20 mM sodium phosphate, pH 7.2, 5 mM $MgCl_2$, and 1 mM DTT. Cross-linking reactions were performed at room temperature by adding 0.002% (final concentration) glutaraldehyde to 0.025 $\mu$g/$\mu$L REGα/REG$\beta$ hetero-oligomers. The reactions were quenched at specified times by adding 60 $\mu$L of 3 M Tris, pH 8.0 for every 440 $\mu$L of reaction mixture. Each of the 500 $\mu$L quenched samples was mixed with 166 $\mu$L of 4× SDS sample buffer and boiled for 10 min. Samples (20 $\mu$L) were applied to 10% SDS−polyacrylamide gels, and electrophoresis was performed at 25 mA/gel (*25*). After electrophoresis, proteins were transferred to nitrocellulose membranes according to a standard procedure (*26*). The membrane was first blocked with 10% dry milk in TBS-0.1% Tween 20 for 2 h and was further blocked with 5% dry milk in TBS-0.1% Tween 20 and 1 M glycine for 1 h. Then anti-REGα or anti-REG$\beta$ specific antiserum was added (1:5000 dilution) to the membrane and chemiluminescence reagents from DuPont NEN were used to visualize the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG from Cappel (1:3000 dilution).

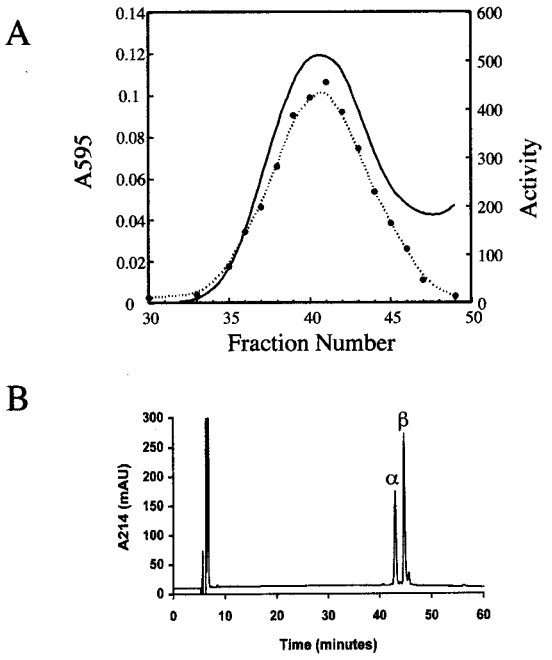
*Determination of the Molecular Mass of REGα/REGβ Hetero-oligomers Using ESI-TOF MS*. Since it is necessary to use volatile buffers such as ammonium bicarbonate or ammonium acetate for ESI (*27*), we first determined whether

REGα(N50Y)/REGβ hetero-oligomers are stable in the ESI buffer used for most of our measurements (10 mM ammonium bicarbonate) by comparing their ability to activate the proteasome in that buffer and in 10 mM Tris buffer normally used for the activity assay (*18*). REGα(N50Y)/REGβ hetero-oligomers (at high protein concentrations) initially activated the proteasome in the ESI buffer to the same extent as in 10 mM Tris buffer, but they lost this ability in the course of time and showed no activity after several hours. Mass spectrometry indicated a similar decline in abundance of the REGα(N50Y)/REGβ large hetero-oligomers in the ESI buffer, although the REGα(N50Y) and REGβ monomers themselves were not degraded (data not shown). These observations suggest that REGα(N50Y)/REGβ gradually loses its ability to activate the proteasome in the ESI buffer because of dissociation of the hetero-oligomer. Consequently, it was necessary to perform all mass measurements immediately after exchange into the ESI buffers.

Samples containing REGα/REGβ hetero-oligomers (~0.1−1 mg/mL equivalent monomer concentration) in 0.5× TSD were shipped on wet ice from Utah to the University of Manitoba for mass analysis. The samples were delivered within 1 day of purification and were analyzed on the day of arrival. The 0.5× TSD buffer was replaced by ammonium bicarbonate buffer immediately prior to mass analysis. Usually part (~350 μL) of the sample was first preconcentrated by a factor of ~7 on a refrigerated centifuge using an ultrafree-MC 10 000 membrane (Millipore Co., Bedford, MA). The buffer was then replaced with ammonium bicarbonate (pH 7.5−7.8) at various concentrations (10−500 mM). The exchange of buffers was performed enough times at 4 °C so that the nonvolatile components of the original buffer were diluted by a factor of at least $10^6$. Part (~50 μL) of the final solution containing ~$10^{-5}$−$10^{-4}$ M REG monomer in 10−500 mM ammonium bicarbonate buffer was immediately examined in an ESI-TOF mass spectrometer (*28*, *29*). Two electrospray ion sources were used. A conventional ESI source, in which the liquid sample is delivered to the spectrometer at a flow rate ~0.2 μL/min, was used for samples in ~10 mM ammonium bicarbonbicarbonate. However, a nanospray (*30*) source (~20 nL/min) was used for some samples that were kept in higher $NH_4HCO_3$ concentrations in order to maintain the structure of the noncovalent complexes.

## RESULTS

*The Apparent Ratio of REGβ to REGα Suggests That the REGα(N50Y)/REGβ Hetero-oligomer Is an α3β4 Heptamer*. We have previously reported that REGα(N50Y), a REGα monomer, forms hetero-oligomers with REGβ, and the resulting hetero-oligomer activates the proteasome to the same extent as does the hetero-oligomer formed from wild-type REGα and REGβ subunits (*19*) (see also Figure 1A). For our intial experiments, we used the REGα(N50Y) mutant because it is a monomer, and the resulting REGα/REGβ hetero-oligomers are not "contaminated" with α7 heptamers. However, as shown below, wild-type REGα also forms heptamers with REGβ when both subunits are coexpressed in *E. coli*. To determine the subunit stoichiometry in REGα(N50Y)/REGβ hetero-oligomers, we purified the hetero-oligomers by sizing chromatography and separated α and β subunits by HPLC (see Figure 1B as an example). Their corresponding peak areas at 214 nm were used to calculate their relative abundance as described in the Experimental Procedures. The apparent molar ratio of REGβ to REGα(N50Y) in the purified hetero-oligomer was 1.4. This value was virtually the same in all fractions (Figure 1C), indicating that a single REGα(N50Y)/REGβ hetero-oligomer species was predominant in the gel filtration eluate. The REGβ to REGα ratios determined in a number of independent experiments were always between 1.2 and 1.5. Similar analyses on 11S REG isolated directly from human red blood cells also produced REGβ to REGα ratios between 1.2 and 1.4 (data not shown). These values suggest that the REGα(N50Y)/REGβ oligomer and natural REG molecules are α3β4 heptamers.

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| Fraction Number | 36 | 38 | 40 | 42 | 44 | Pooled |
|---|---|---|---|---|---|---|
| β/α ratio | 1.43 | 1.45 | 1.45 | 1.45 | 1.46 | 1.44 |

FIGURE 1: Subunit composition of hetero-oligomers formed from a monomeric REGα varient (N50Y) and wild-type REGβ. (A) Gel filtration profile of REGα(N50Y)/REGβ hetero-oligomers (solid line). Equal amounts of purified REGα(N50Y) and REGβ were mixed, incubated at 4 °C and subjected to gel filtration analysis on Superdex 200 in a Pharmacia HiLoad 26/60 column (solid line). Selected fractions were used to perform proteasome activation assays in 10 mM Tris buffer, pH 7.5 (dashed line). (B) HPLC analysis of REGα(N50Y)/REGβ hetero-oligomers. REGα(N50Y)/REGβ hetero-oligomers were loaded onto a C18 column, and REGα(N50Y) was separated from REGβ as described in the Experimental Procedures. (C) Apparent REGβ to REGα(N50Y) molar ratios. REGα(N50Y) and REGβ in each of the fractions from gel filtration chromatography were separated on a HPLC column as shown in panel B, and the ratio of REGβ to REGα was calculated as described in the Experimental procedures. Pooled: All the peak fractions in panel A were pooled together, concentrated and dialyzed against 0.5× TSD before HPLC analysis.

*Chemical Cross-Linking of Hetero-oligomers Produced REGβ−REGβ Dimers*. Song et al. (*22*) chemically cross-linked PA28 and observed identical patterns of cross-linked

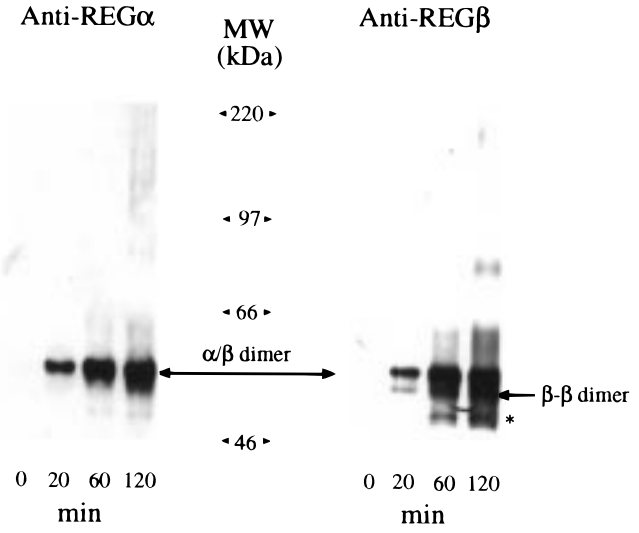


FIGURE 2: Chemical cross-linking of REGα(N50Y)/REG$\beta$ hetero-oligomers. REGα(N50Y)/REG$\beta$ hetero-oligomers were partially cross-linked using glutaraldehyde in phosphate buffer as described in the Experimental Procedures, and cross-linked products were separated on SDS gels, transferred to nitrocellulose membranes, probed with REGα specific serum (left panel) or with REG$\beta$ serum (right panel) and visualized by chemilumilescent reagents. The blunt arrows identify the migration positions of molecular weight markers. The cross-linking reactions were stopped after 20, 60, or 120 min and time "0" refers to a sample lacking glutaraldehyde. The band labeled by an asterisk (*) revealed by anti-REG$\beta$ serum may be a degradation product of the REG$\beta$−REG$\beta$ dimer or it may represent REG$\beta$−REG$\beta$ dimers with changed mobility on a SDS−polyacrylamide gel due to multiple cross-links.

products upon staining with either REGα or REG$\beta$ specific antisera. On the basis of these results, they proposed that PA28 is a hexamer in which α and $\beta$ subunits alternate. However, for the reasons mentioned above, we suspected that α/$\beta$ hetero-oligomers would prove to be heptamers. If REGα(N50Y)/REG$\beta$ hetero-oligomers are indeed α3$\beta$4 heptamers, there should be at least one REG$\beta$−REG$\beta$ interface in the complex. Moreover, if six of the α and $\beta$ subunits alternate, just two kinds of dimers, α−$\beta$ and $\beta$−$\beta$, should appear. To test this possibility, we exposed hetero-oligomers to glutaraldehyde under conditions where dimers would be the major cross-linked products. We then subjected the cross-linking reactions to SDS−PAGE and analyzed the products using REGα and REG$\beta$ specific sera. After short electrophoretic runs such that REGα separated slightly from REG$\beta$, the cross-linked products detected by REGα serum were identical to those revealed by anti-REG$\beta$ (data not shown). This observation agrees with the results of Song et al (*22*). However, clear differences were observed when electrophoresis was extended to the point that the 46 K molecular weight marker migrated to the bottom of the gel. Although a single cross-linked product was revealed by the REGα specific serum, two closely spaced cross-linked products were evident after staining with anti-REG$\beta$ serum (Figure 2). The mobility of the slower anti-$\beta$ reactive species corresponded to that seen after staining with anti-REGα, indicating that it was an α−$\beta$ dimer; the slightly faster migrating species, which we take to be a $\beta$−$\beta$ dimer, was only recognized by anti-REG$\beta$ serum. As can be seen in Figure 2, the slower species stains more intensely than the faster one. These relative abundances are expected if the REGα(N50Y)/REG$\beta$ hetero-oligomer is a heptamer with

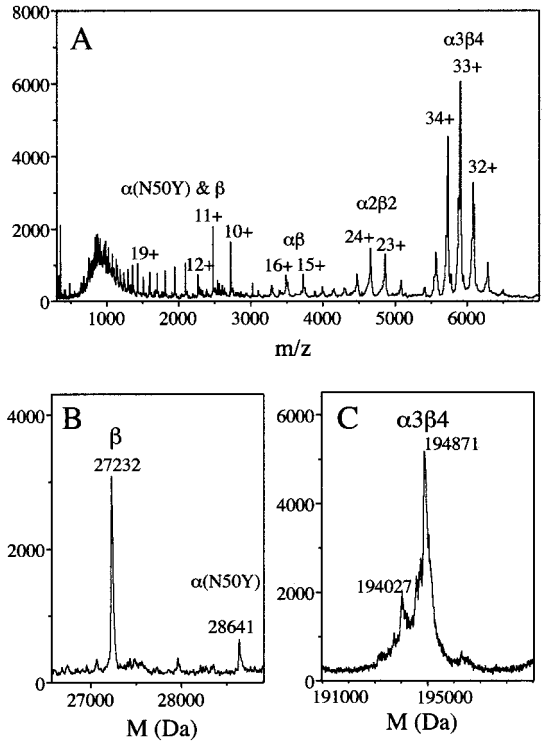


FIGURE 3: Electrospray mass spectrometric analyses of REGα-(N50Y)/REG$\beta$ and REGα(N50Y)/REG$\beta\Delta$9 hetero-oligomers. (A) Mass over charge (*m*/*z*) spectrum of REGα(N50Y)/REG$\beta$ hetero-oligomers. (B) Deconvoluted spectra of the region corresponding to *m*/*z* between 1000 and 3000. This region identifies REGα(N50Y) and REG$\beta$ monomers. The $\beta$ subunit peak is much larger than the α subunit peak because there is an extra $\beta$ subunit in the α3$\beta$4 heptamer and most dissociation products were greater than or equal to α$\beta$ dimers. (C) Deconvoluted spectra of the region around *m*/*z* 6000.

three alternating α$\beta$ dimers and an extra $\beta$ subunit since cross-linking of such a heptamer would produce more α−$\beta$ dimers than $\beta$-$\beta$ dimers. The existence of an apparent $\beta$−$\beta$ dimer and its relative abundance provide additional evidence that REGα(N50Y)/REG$\beta$ hetero-oligomers are α3$\beta$4 heptamers.

*ESI-TOF Mass Spectrometry Demonstrates That REGα/REG$\beta$ Hetero-oligomers Are Heptamers*. Electrospray ionization (ESI) (*27*) is a powerful technique for determining the masses of biomolecules. Because it is a relatively gentle mode of ionization, it has been particularly useful for the study of noncovalent interactions and the stoichiometry of protein complexes (*31*, *32*). An ESI source may be coupled to various types of mass analyzer, but time-of-flight (TOF) mass spectrometers have an effectively unlimited mass to charge number (*m*/*z*) range, an important advantage for observing noncovalent complexes (*33*) especially those of large mass (*34*, *35*). Accordingly, an ESI-TOF MS instrument (*28*, *29*) was chosen to measure the molecular mass of the REGα(N50Y)/REG$\beta$ hetero-oligomer.

Figure 3A shows a typical ESI TOF MS spectrum from the REGα(N50Y)/REG$\beta$ hetero-oligomers. Four different charge state distributions were observed with *m*/*z* values around 6000, 4500, 3500, and 1000−3000. Deconvolution (*36*) of the *m*/*z* distribution from 1000 to 3000 yielded the mass spectrum shown in Figure 3B, which contains two major species. These have masses in excellent agreement with the masses calculated for recombinant REG$\beta$ and

Table 1: Mass Spectrometry of REG Hetero-oligomers Formed from REGα(N50Y) and REG$\beta$ or REG$\beta\Delta$9[a]

| species | theoretical mass (Da) | experimental mass (Da) | relative abundance in the spectrum |
|---|---|---|---|
| REGα(N50Y) | 28 643 | 28 641 ± 6 | 0.12 |
| REG$\beta$ | 27 232 | 27 232 ± 6 | 0.61 |
| α$\beta$ | 55 875 | 55 884 ± 11 | 0.14 |
| α2$\beta$2 | 111 749 | 111 755 ± 22 | 0.24 |
| α3$\beta$4 | 194 856 | 194 871 ± 40 | 1.00 |
| REGα(N50Y) | 28 643 | 28 641 ± 6 | 0.43 |
| REG$\beta\Delta$9 | 26 182 | 26 182 ± 6 | 1.00 |
| α$\beta\Delta$9 | 54 825 | 54 838 ± 11 | 0.15 |
| α2($\beta\Delta$9)2 | 109 649 | 109 668 ± 22 | 0.12 |
| α3($\beta\Delta$9)4 | 190 655 | 190 682 ± 40 | 0.11 |

[a] The theoretical mass of each recombinant subunit without the first methionine was calculated as described in the Experimental Procedures. The theoretical masses of the various hetero-oligomers were, in turn, calculated from the theoretical mass of each recombinant subunit. The experimental masses were obtained by ESI-TOF MS. The relative intensities varied considerably from run to run, but typical values are given here and in the other tables.

REGα(N50Y) monomers (Table 1). Similar deconvolutions for *m*/*z* ~3500 and ~4500 (not shown) indicate the presence of an α$\beta$ dimer and an α2$\beta$2 tetramer, respectively (Table 1). Deconvolution of the most abundant distribution (*m*/*z* ~6000) produced the mass spectrum in Figure 3C, which exhibits a predominant peak at 194 871 Da, in good agreement with the theoretical mass (194 856 Da) of a REGα(N50Y)/REG$\beta$ hetero-oligomer containing three α and four $\beta$ subunits (Table 1). Several minor components appeared between 192 and 195 kDa, probably resulting from minor degradation or modifications in the REGα(N50Y) or REG$\beta$ subunits. In fact, a species with a mass of 26 368 was observed in the ESI system of denatured proteins, and this mass is within 1 Da of that expected from a REG$\beta$ subunit missing the last seven residues (calculated mass 26 367 Da). But it is important to note that there was no sign of a REGα/REG$\beta$ hexamer (calculated mass 167 625 Da). On the other hand, there was a very small peak at ~196 300 Da, consistent with the presence of a complex consisting of four α and three $\beta$ subunits (theoretical mass 196 268 Da, see additional results below). Summing the contributions to the mass spectrum from free monomers and the monomer content of the oligomers resulted in an apparent REG$\beta$/REGα ratio of ~1.4 for the sample analyzed in Figure 3.

Previously, we have shown that REGα(N50Y) forms hetero-oligomers with REG$\beta\Delta$9, a REG$\beta$ subunit lacking its last nine amino acids, and the apparent molar ratio of REG$\beta\Delta$9 to REGα(N50Y) in these complexes (1.2 to 1.4) is very similar to the ratio observed for hetero-oligomers containing wild-type $\beta$ subunits (*21*). Because the mass of each mutant $\beta$ subunit is 1050 Da less than a wild-type REG$\beta$ subunit, an α3$\beta$4 heptamer formed from REG$\beta\Delta$9 subunits should be 4 200 Da smaller than the hetero-oligomer formed by REGα(N50Y) and REG$\beta$. With this in mind, we examined the mass of REGα(N50Y)/REG$\beta\Delta$9 hetero-oligomers by ESI-TOF MS. The *m*/*z* spectrum was similar to that shown for REGα(N50Y) and REG$\beta$ in Figure 3A except that the abundance of ions with *m*/*z* about 6000 was considerably lower. Nevertheless, monomers, dimers, tetramers, and heptamers were present, with masses in good agreement with their calculated values (Table 1). In particular, the heptamer mass observed at 190 682 Da is almost exactly 4200 Da less than that recorded for the REGα3/REG$\beta$4 heptamer as predicted. Thus, the mass measurements on REGα/REG$\beta\Delta$9 complexes provide further evidence that the preferred hetero-oligomer is an α3$\beta$4 heptamer.

Table 2: Molecular Masses of Wild-type REGα/REG$\beta$ Hetero-oligomers in Which the $\beta$/α Ratio Was ~1.2[a]

| species | theoretical mass (Da) | experimental mass (Da) | relative abundance in the spectrum |
|---|---|---|---|
| REGα | 28 592 | 28 593 ± 6 | 0.15 |
| REG$\beta$ | 27 232 | 27 232 ± 6 | 0.50 |
| α$\beta$ | 55 824 | 55 828 ± 11 | 0.07 |
| α2$\beta$2 | 111 648 | 111 662 ± 22 | 0.10 |
| α3$\beta$4 | 194 704 | 194 740 ± 40 | 1.00 |
| α4$\beta$3 | 196 064 | 196 112 ± 40 | 0.16 |

[a] Plasmids encoding wild-type REGα and REG$\beta$ were cotransformed and expressed in *E. coli* as described in the Experimental Procedures. The resulting recombinant proteins were purified and analyzed by an ESI-TOF MS.

To obtain more information about the structure of the heptameric complex, we have measured the *m*/*z* spectrum as the "declustering voltage" between the capillary and the skimmer in the ESI source is increased (*28*, *33*), so as to break up the complex by collisions with the ambient gas molecules. The heptamer is first observed to break up into a 3α/3$\beta$ hexamer (plus a $\beta$ monomer). At still higher declustering voltage, the heptamer disappears altogether, but the hexameric peaks are still present, suggesting that the extra $\beta$ subunit in the heptamer is bound a good deal more weakly than the other components in the structure in the gas phase (data not shown). However, it is not clear how closely this is related to its behavior in solution.

*Hetero-oligomers Containing Wild-Type REGα and REG$\beta$ Subunits Are Also Heptamers*. As the experiments described above were performed with the variant REGα(N50Y), it was clearly important to determine whether wild-type REGα also forms a heptamer with REG$\beta$. For this purpose, we coexpressed wild-type REGα and REG$\beta$ in *E. coli* cells and purified the recombinant hetero-oligomers. Two samples with different REG$\beta$ to REGα ratios were produced. For one sample, the REG$\beta$ to REGα ratios were ~1.2 in all fractions across the gel filtration peak. The ESI-TOF MS spectrum from this sample was quite similar to the spectrum from REGα(N50Y)/REG$\beta$ (data not shown). Again there were four charge state distributions, with *m*/*z* values from 1000 to 2000 and around 4000, 5000, and 6000. Deconvolution yields the masses shown in Table 2. The ions with *m*/*z* 1000−2000 arise from REGα and REG$\beta$ monomers and the ions around *m*/*z* 4000 and 5000 from α−$\beta$ dimers and α2$\beta$2 tetramers, respectively. Deconvolution of the *m*/*z* spectrum around 6000 yields the mass spectrum shown in Figure 4A, where the mass of the major peak was in very good agreement with the mass calculated for an α3$\beta$4 heptamer. REG α4$\beta$3 heptamers were also present in this sample and at higher concentration than in the spectrum of the REGα(N50Y)/REG$\beta$ hetero-oligomer. In addition, there were several minor species, possibly arising by deletion of a few amino acid residues from the constituent monomers. However, once again there was no sign of hexamers in the mass spectrum.

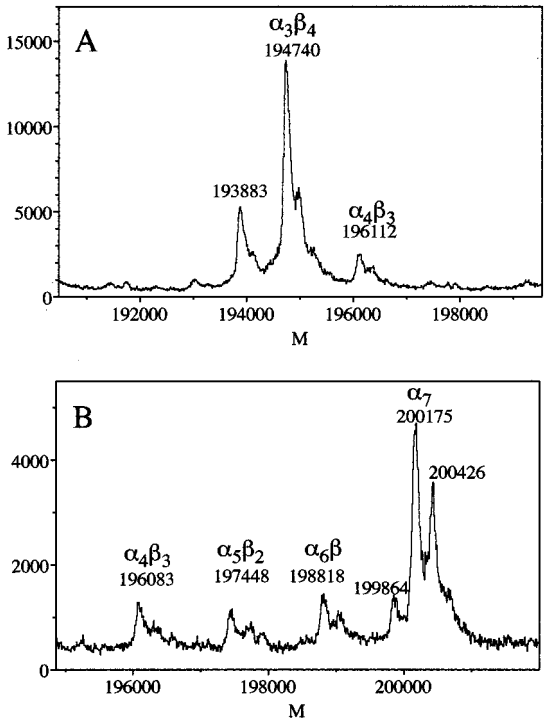


FIGURE 4: Mass spectra of hetero-oligomers formed from wild-type REGα and REG*β* subunits. (A) The deconvoluted spectrum of the region corresponding to *m*/*z* about 6000 is shown from a sample containing REG*β* and REGα at an apparent ratio of 1.2. Heptamers α3*β*4 and α4*β*3 were observed, with α3*β*4 being the predominant one. Wild-type REGα and REG*β* were coexpressed in bacteria *E. coli* and recombinant proteins were purified and subjected to ES-TOF MS analysis as described in the Experimental Procedures. (B) The deconvoluted spectrum of heptameric regions is shown from a sample in which the apparent molar ratio of REG*β* to REGα was 0.1. Heptamers α7, α6*β*, α5*β*2, and α4*β*3 were observed.

For the second sample, the REG*β* to REGα ratios varied from 0.1 to 0.15 across the gel filtration peak. REGα and REG*β* subunits were again observed, but α3 and α2*β* trimers were present rather than the α2*β*2 tetramers seen when the *β*/α ratio was greater than 1 (data not shown). More interestingly, there were four kinds of heptamers α7, α6*β*, α5*β*2, and α4*β*3, with the α7 heptamer being the predominant species (see Figure 4B). No clear indication of an α3*β*4 heptamer was observed, presumably because of the shift in equilibrium caused by the limited amounts of REG*β* in the sample. The observed masses are shown in Table 3. Minor components were also present, as well as a fairly large peak at 200 426 Da, whose mass agrees with the theoretical mass of an α7 heptamer with each subunit acetylated (200 445 Da). The apparent existence of a heptamer containing only acetylated subunits might be considered surprising, but there is a species in the monomeric region with the mass expected from an acetylated REGα monomer.

## DISCUSSION

We have presented evidence that recombinant REGα and REG*β* subunits preferentially form hetero-oligomers that contain seven, not six, subunits. The apparent ratio of REG*β* to REGα subunits in recombinant α/*β* hetero-oligomers was consistently found in the narrow range 1.2−1.5 (Figure 1). These experimentally determined stoichiometries closely approximate the value of 1.33 expected from a REG hetero-oligomer containing 3 α and 4 *β* subunits. The second piece of evidence favoring heptamers was obtained by cross-linking recombinant hetero-oligomers. Glutaraldehyde treatment of REGα(N50Y)/REG*β* complexes generated a product with the electrophoretic mobility, antigenic properties, and abundance expected from a REG*β*−REG*β* dimer; the corresponding REGα/REGα dimer was not observed (Figure 2). Neither of these findings can be considered compelling evidence for the heptamer model. The apparent ratio of REGα to REG*β* could be affected by differential recovery of α and *β* subunits from the C18 column as well as by comigrating contaminant proteins. Likewise, the cross-linking results might be viewed skeptically. Identification of the faster migrating band in Figure 2 as a *β*−*β* dimer can be questioned since the electrophoretic properties of cross-linked proteins cannot be predicted with great certainty. Nonetheless, the mass measurements (Tables 1−3) strongly support our interpretations of both the cross-linking results (Figure 2) and inferences derived from REG*β*/REGα ratios (Figure 1). Furthermore, it should be noted that cross-linking analyses (*22*) and measurements of the relative abundance of REGα and REG*β* subunits (*23*) are the very same techniques used to arrive at the hexamer model.

Table 3. Molecular Masses of Wild-type REGα/REG*β* Hetero-oligomers in Which the *β*/α Ratio Was ∼0.1[a]

| species | theoretical mass (Da) | experimental mass (Da) | relative abundance in the spectrum |
|---|---|---|---|
| REGα | 28 592 | 28 592 ± 6 | 1.00 |
| REG*β* | 27 232 | 27 232 ± 6 | 0.12 |
| α*β* | 55 824 | 55 828 ± 11 | 0.04 |
| α2 | 57 184 | 57 184 ±11 | 0.03 |
| α2*β* | 84 416 | 84 413 ± 15 | 0.01 |
| α3 | 85 776 | 85 776 ± 15 | 0.02 |
| α4*β*3 | 196 064 | 196 083 ± 15 | 0.10 |
| α5*β*2 | 197 424 | 197 448 ± 40 | 0.08 |
| α6*β* | 198 784 | 198 818 ± 40 | 0.09 |
| α7 | 200 144 | 200 175 ± 40 | 0.40 |

[a] Plasmids encoding REGα and REG*β* were sequentially transformed into *E. coli* and coexpressed as described in the Experimental Procedures. The resulting hetero-oligomers were purified and analyzed by ESI-TOF MS.

By far the best evidence that recombinant REGα/*β* hetero-oligomers contain seven subunits was obtained using ESI-TOF mass spectrometry. Almost all of the experimentally determined masses greater than 120 kDa are in excellent agreement with those expected from heptamers. Indeed, the observed masses were, in every case, within 50 Da of the masses calculated for heptamers. REG heptamers, with masses of about 200 000 Da, are among the largest complexes successfully measured by ESI-TOF MS, and the observed deviations from expected masses are less than 0.03% (Tables 1−3). Heptamers were observed in all hetero-oligomer combinations tested: REGα(N50Y)/REG*β*, REGα-(N50Y)/REG*β*Δ9, and REG*β*/REGα at ratios of 1.2 or 0.1. Moreover, with a single exception, heptamers were the only observed REGα/*β* hetero-oligomers found containing more than four subunits. A species with the mass expected for a hexamer was observed (along with monomers, dimers, tetramers, and heptamers) in only one spectrum, taken from a sample that sat overnight at pH 8.5. It is possible that hexamers can form under nonphysiological conditions, presumably by dissociation of heptamers.

The presence of monomers, dimers and other small oligomers in the various spectra raises the possibility that hexamers were present in the hetero-oligomer populations, but they dissociated during the mass measurements. Although this possibility cannot be rigorously excluded, it is unlikely in view of the stability of the α3β3 hexamers intentionally generated by collision of heptamers with ambient gas molecules (see Results). Moreover, in the spectrum of Figure 3A, for example, the heptamer peaks contain 70% of all ions detected. Even if all the other ions arose from dissocation of hexamers, there could only have been one hexamer for every seven heptamers in the original solution.

The mass spectra in Figure 4B show that when the REGβ/REGα ratio was 0.1, four different hetero-oligomers (α7, α6β, α5β2, and α4β3) were produced. This contrasts with the predominant formation of an α3β4 heptamer when the β/α ratio was 1.2 (Figure 4A). We offer the following explanation for these observations. We assume that α3β4 is the most stable heptamer that can be formed from recombinant REGα and REGβ subunits. Its absence from the mass spectrum in Figure 4B can be explained by the large excess of REGα subunits. Because REGα binds REGβ more tightly than it binds itself (*11*), the α2β trimers seen in Table 3 are very likely arranged α−β−α. Although these trimers arise by dissociation of heptamers, α−β−α trimers may well form during the initial assembly of REGα/REGβ hetero-oligomers. At high REGα concentrations, the α−β−α trimer might sequester free β monomers, thereby preventing the β−β contact needed to generate an α3β4 heptamer. Thus, although α3β4 heptamers appear to be the most stable hetero-oligomers formed from recombinant REGα and REGβ subunits (Figure 4A), we suggest that they simply cannot assemble in the presence of a vast excess of REGα subunits. We did not observe heptamers containing more than four β subunits in any of the samples analyzed. An α2β5 heptamer requires three adjacent β subunits in the ring, and REGβ only forms trace amounts of dimers in solution. Thus, the β subunit may be unable to form the homotrimer required for formation of an α2β5 heptamer (*11*). Alternatively, the concentrations of REGβ may not have been high enough to allow formation of heptamers containing five or more more β subunits.

Whereas it seems clear that recombinant REGα and REGβ form heptameric complexes, we have been unable to resolve the masses of 11S REG molecules isolated directly from red blood cells. The problem stems both from the difficulty in obtaining reasonable amounts of pure red cell 11S REG and from considerable heterogeneity in monomer molecular weights for α and β subunits comprising the molecule purified from red cells. For example, distinct peaks with masses of 26 520, 26 583, and 26 895 Da were observed, the first and last of these match values calculated for REGβ subunits missing eight and five amino acids, respectively, from their N-termini. Likewise, we observed a series of masses starting at 28 120 Da and increasing in increments of ∼32 to 28 216 Da. The mass 28 120 Da would be generated by a human REGα subunit lacking 5 N-terminal residues. The three species larger than 28 120 Da could represent oxidation products since 11S REG is purified from oxygen-rich red blood cells. In any event, repeated attempts to measure the oligomeric state of red blood cell 11S REG by ESI-TOF MS have failed.

Despite the absence of direct mass measurements on red cell 11S REG, we offer several arguments to support the idea that the natural proteasome activator is also a heptamer. First, an extensive comparison of recombinant REGα and human red cell 11S REG showed that the two molecules are virtually equivalent proteasome activators (*37*). Since recombinant REGα has been shown to be a heptamer by X-ray diffraction (*17*) and ESI-TOF MS (Figure 4B), it seems unlikely that a *hexameric* α/β hetero-oligomer would have the same biochemical properties as the recombinant heptamer. Second, direct measurements of the apparent β/α stoichiometry in red cell 11S REG almost always produced ratios ≥1.2 in good agreement with the data from recombinant α/β heptamers presented in Figure 1. Third, the results in Figures 3 and 4 clearly show that recombinant REGα and REGβ subunits preferentially form mixed heptamers. We would be surprised to find that synthesis in eukaryotic cells would change the inherent affinities of the subunits for one another. Still, it must be admitted that posttranslational modifications to REGα and REGβ subunits could affect oligomerization. Or, eukaryotic chaperonins might direct assembly of α/β hexamers rather than heptamers. For these reasons, we cannot rigorously exclude the possibility that 11S REG from eukaryotic cells is a hexamer. Nonetheless, at the very least our results cast serious doubt on the hexamer model. In fact, we believe that the experiments presented above strongly support heptamer models for both recombinant and natural 11S REG complexes.

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