Human lymphoblast and erythrocyte multicatalytic proteases: differential peptidase activities and responses to the 11S regulator

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Abstract The multicatalytic protease (MCP) or 20S proteasome was purified from human red blood cells and two lymphoblastoid cell lines, 721.45 which constitutively expresses protease subunits LMP2 and LMP7, and 721.174 in which genes for these subunits are deleted. Each MCP was assayed using a series of fluorogenic peptides. The hydrophobic peptides gGGF-MCA, sRPFHLLVY-MCA and sLY-MCA were particularly good substrates for 721.45 MCP as compared to the enzyme from 721.174 and red blood cells. In addition, hydrolysis of gGGF-MCA and sLY-MCA was activated by human red blood cell and recombinant regulators to a greater extent using MCP from 721.45 lymphoblasts. Thus, LMP2/LMP7 and regulator appear to act synergistically in the enhanced degradation of gGGF-MCA and sLY-MCA by the multicatalytic protease.

Key words: Proteasome; LMP2; LMP7; IFNγ-induced regulator; Antigen presentation

1. Introduction

The multicatalytic protease (MCP) or 20S proteasome is a large multisubunit enzyme abundant in the nucleus and cytoplasm of eukaryotic cells [1–3]. MCP has a native molecular weight of ~700 kDa, and in eukaryotes it is composed of at least 14 distinct subunits with molecular masses between 21 and 32 kDa [4]. The subunits are arranged in four rings each containing 7 subunits; the rings stack upon one another to form a cylindrical structure [5]. The multicatalytic protease exhibits at least five endopeptidase activities that cleave bonds at the carboxyl side of basic, hydrophobic-neutral and acidic amino acids [6]. It has long been suspected that the active sites are buried within the cylinder [3], and the recently reported X-ray structure of the *Thermoplasma* proteasome confirms this suspicion [7].

MCP is believed to be responsible for the degradation of many nuclear and cytosolic proteins. It has been demonstrated that the multicatalytic enzyme associates with two additional multisubunit complexes. One is an 11S regulator which in red blood cells is composed of two distinct ~30 kDa subunits [8–11]. Binding of the 11S regulator with MCP can enhance MCP's peptidase activity as much as 60-fold. The other is a regulatory complex composed of at least 15 different subunits [12–16]. Association of MCP with the regulatory complex produces the 26S protease which is responsible for ATP-dependent degradation of ubiquitin-conjugated proteins. As part of the larger 26S enzyme, MCP is clearly involved in key cellular functions such as cell cycle traverse and selective intracellular proteolysis (for reviews, see [17–19]).

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Two MCP subunits, LMP2 and LMP7, are encoded in the MHC-class II genomic region [20,21], and their expression is upregulated by IFNγ [22]. These findings suggest that MCP may be involved in the processing of cellular proteins for MHC-class I antigen presentation. Supporting the potential role of MCP in class I-mediated immune responses is the fact that synthesis of an 11S regulator subunit is also stimulated by IFNγ [23]. Conceivably, the subunit composition of MCP and its response to the regulator influence the production of peptides for presentation on MHC-class I molecules [24,25]. Here, we show that MCP from human lymphoblasts expressing LMP2 and LMP7 hydrolyzes several fluorogenic peptides faster than MCP from a derivative cell line lacking the MHC II-encoded subunits. Moreover, both red blood cell and recombinant regulators enhance cleavage of these peptides by MCP containing LMP2 and LMP7 to a greater extent than they stimulate the enzyme from mutant lymphoblasts.

2. Materials and methods

MCP and 11S regulator were prepared from 20 units of outdated human red blood cells following the protocols of Dubiel et al. [10,26]. MCPs from human lymphoblastoid cell lines 721.45 and 721.174 were purified as described [27] except that a 55–80% ammonium sulfate precipitation step was introduced between DEAE chromatography and glycerol gradient centrifugation. Recombinant regulator was purified from *Escherichia coli* after induction with IPTG. Briefly, the cells were lysed using a french press and the lysate passed over an anion exchange column followed by sizing chromatography.

MCP peptidase activity was assayed as follows: 200-ng aliquots of each purified MCP were incubated at 37°C with 100 μM fluorogenic peptide in 100 μl of 50 mM Tris, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM Na EDTA, 1 mM DTT, pH 7.8. Duplicate samples were quenched after 20 and 40 min by adding 200 μl of ice-cold ethanol. Fluorescence was determined in a Perkin-Elmer LS-5 fluorimeter at 380 nm excitation/440 nm emission wavelengths for MCA and 335 nm excitation//410 nm emission wavelengths for β-NA peptides. Human RBC regulator (2.6 μg) was added to the reaction mixtures containing MCPs; alternatively, 3.6 μg of recombinant regulator was added. Samples were preincubated in the presence of regulator for 10 min at 37°C before adding substrate. Specific activities are expressed as pmol/min/μg of MCP.

3. Results

MCPs were isolated from human red blood cells and the two human lymphoblastoid lines, 721.45 and 721.174. In addition, human 11S regulator was partially purified from red blood cells and the recombinant regulator, containing just the 29 kDa subunit, was purified from *E. coli*. The purity of each component was assessed by electrophoresis on 10% SDS-PAGE gels (Fig. 1). The purified multicatalytic proteases were incubated with 21 different fluorogenic peptides. Cleavage was <1 pmol/min/μg for fluorogenic peptides with a proline in position P1

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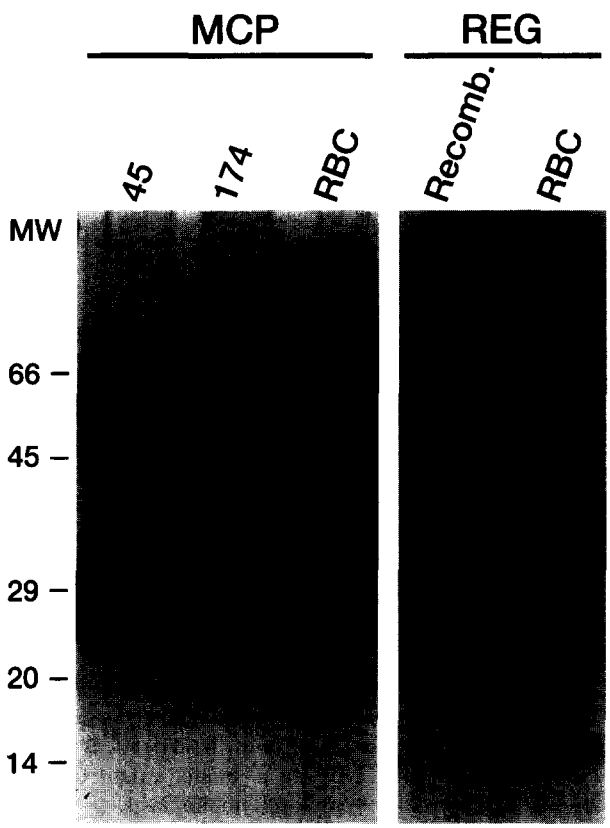

Fig. 1. SDS-PAGE analysis of MCPs and regulators. Proteins were purified as described under experimental procedures and separated on a 10% polyacrylamide gel followed by silver staining.

or P2 as well as for boc-EKK-MCA, cbz-RRR-MCA, boc-IEGR-MCA and suc-AAA-MCA. Specific activities for the remaining fluorogenic peptides are listed in Table 1. In general, the multicatalytic protease from human RBCs cleaved fluorogenic peptides at a lower rate than the enzyme from lymphoblasts, and except for cLLE-βNA, most fluorogenic peptides were hydrolyzed faster by 721.45 MCP than by 721.174 MCP. The most striking differences between 721.45 and 721.174 MCPs were observed with three hydrophobic peptides, sRPFHLLVY-MCA, gGGF-MCA and sLY-MCA. The multicatalytic protease containing LMP2 and LMP7 (721.45 cells) hydrolyzed these peptides 3–7-fold faster than MCP from 721.174 cells.

Fluorogenic peptides with identical amino acids at the P1 position varied markedly as substrates (see Table 1). For example, rates of hydrolysis for two peptides with tyrosine at the P1 position differed by 20-fold using the red blood cell enzyme. In addition, hydrolysis of four peptides with arginine at the P1 position varied as much as 5-fold using each of the enzymes. Thus, in agreement with a number of other studies [6,28,29], we found that the amino acid residue at the P1 position does not uniquely determine rates of peptide hydrolysis by MCP.

We also assayed each MCP in the presence of red blood cell or recombinant regulators. Equivalent saturation curves were obtained upon incubation of MCP from each cell type with increasing amounts of red blood cell regulator (Fig. 2). The recombinant regulator produced similar results except that greater amounts of protein (3.6 μg) were required to reach saturation (data not shown). MCP activity was reassayed against the panel of fluorogenic substrates in the presence of saturating amounts of red blood cell or recombinant regulator. It is evident from the data in Table 2 that hydrolysis of most peptides was activated to about the same extent with each enzyme. However, cleavage of gGGF-MCA and sLY-MCA was stimulated more using 721.45 MCP and hydrolysis of two substrates with basic residues at the P1 position, FSR-MCA and bVLK-MCA, was stimulated almost 2-fold greater using MCP from 721.174 cells. The results in Table 2 also demonstrate that recombinant and red blood cell regulators stimulate cleavage of specific peptides in a very similar manner. Red blood cell or recombinant regulators did not promote hydrolysis of any peptide that was not already a substrate (data not shown).

4. Discussion

We previously reported that MCP from 721.45 cells hydrolyzes gGGF-MCA more efficiently than MCP from 721.174 cells [27]. The experiments presented above extend these findings to sLY-MCA and sRPFHLLVY-MCA as well (Table 1). Except for the absence of LMP2 and LMP7 subunits in mutant cell lines, two-dimensional gel patterns of MCP components are very similar among mutant and parental lymphoblast lines (e.g. T1 vs. T2 [30]). For this reason, we consider it reasonable to attribute the observed differences in enzymatic activity between 721.45 and 721.174 MCPs to the LMP2 and/or LMP7 subunits. If this assumption is correct, then the ratio of sLLVY-MCA to sLY-MCA cleavage could prove to be a useful measure of the presence of LMP2 and/or LMP7 subunits in proteasomes. It is apparent from the data in Table 1 that this ratio is 3 for MCP from 721.45 cells, 12 for MCP from 721.174 cells, and 19 for MCP from red blood cells. A ratio <5 could imply the presence of LMP2 and LMP7, whereas a ratio >10 may indicate the absence of the two subunits. In any event, the data in Table 1 demonstrate that there are clear differences in the patterns of peptide hydrolysis by MCPs from 721.45 and 721.174 lymphoblasts.

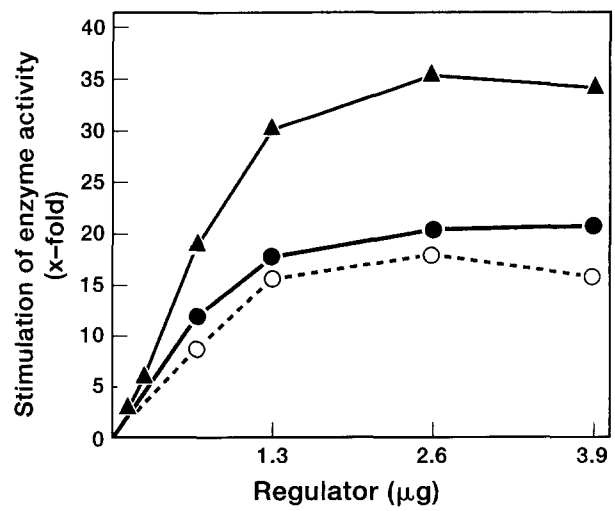

Fig. 2. Activation of sLLVY-MCA cleavage by red blood cell regulator. The graph shows regulator-dependent activation of peptide hydrolysis by MCPs from 721.45 cells (○), 721.174 cells (●) and RBCs (▲).

Table 1
Substrate specificities of lymphoblast and red blood cell multicatalytic proteases

Substrate	Specific activities (pmol/min/μg)		
	RBC-MCP	721.45 MCP	721.174 MCP
Basic			
Boc-FSR-MCA	5 ± 2 (n = 8)	16 ± 4 (n = 6)	8 ± 2 (n = 4)
PFR-MCA	3 ± 1 (n = 7)	9 ± 3 (n = 9)	10 ± 4 (n = 6)
Boc-VLK-MCA	1 ± 0.2* (n = 8)	5 ± 1 (n = 6)	3 ± 1 (n = 6)
Boc-EGR-MCA	4 ± 2 (n = 8)	3 ± 1 (n = 8)	2 ± 0.3 (n = 4)
Boc-GKR-MCA	1 ± 0.6* (n = 8)	3 ± 1 (n = 4)	2 ± 1 (n = 6)
Acidic			
N-Cbz-LLE-βNA	29 ± 10 (n = 8)	33 ± 5 (n = 5)	61 ± 11 (n = 6)
Hydrophobic			
Suc-LLVY-MCA	58 ± 10 (n = 6)	145 ± 18 (n = 4)	92 ± 24 (n = 4)
Suc-RPFHLLVY-MCA	5	18	6
Suc-AAF-MCA	6 ± 3 (n = 8)	40 ± 13 (n = 8)	21 ± 2 (n = 4)
Glu-GGF-MCA	4 ± 1 (n = 8)	30 ± 6 (n = 6)	11 ± 1 (n = 6)
Suc-LY-MCA	3 ± 1 (n = 8)	51 ± 5 (n = 4)	8 ± 1 (n = 4)

*Values <1 pmol/min/μg are below the limit of detection. Peptides are identified using the one-letter amino acid code.

Both regulator and the LMP subunits promote rapid hydrolysis of gGGF-MCA and sLY-MCA (Table 2). Because regulator and LMP subunits are induced by IFNγ [22,23], the observed synergism supports the idea that IFNγ induction serves to alter the kinetic properties of MCP. One can speculate that IFNγ induction of the three components shifts cleavage sites in natural precursors such that the resulting peptides are less 'self-like' or have C-termini appropriate for binding MHC-class I molecules. This inference may seem at odds with a previous paper from this laboratory where we called into question claims that IFNγ alters the kinetic properties of purified proteasomes [31]. We consider both conclusions compatible. In our earlier studies (27) exposure of 721.45 or 721.174 human lymphoblasts to IFNγ did not markedly alter the enzymatic activities of *purified* proteasomes from either line. Still, there are major differences in hydrolysis of fluorogenic peptides between the two cell lines, and these differences are magnified by the 11S regulator. Since IFNγ induces synthesis of the regulator subunit [23], the cytokine may well indirectly influence selection of cleavage sites by the multicatalytic protease.

All the data presented above were obtained using fluorogenic peptides. It can be argued that MCA-peptides are artificial substrates and therefore have no bearing on the intracellular production of peptides for immune presentation. The same arguments can be used to evaluate other studies where longer, non-fluorogenic peptides have been employed [32–34]. The nature of endogenous precursors to presented peptides remains unclear (e.g. folded proteins or nascent chains?, long peptides or short ones?, ubiquitin peptides?, etc.). Furthermore, the small central cavity formed by the two β rings [7] would seem to require that substrates are unfolded at the time of peptide bond hydrolysis. Accordingly, we believe it is reasonable to infer that the differences in MCP activities revealed by fluorogenic peptides reflect inherent properties of the enzyme that determine cleavage site selection in natural substrates.

In summary, we have shown that multicatalytic proteases from human red blood cells and human lymphoblasts hydrolyze specific fluorogenic peptides at significantly different rates. MCP containing LMP2 and LMP7 subunits (721.45 cells) is particularly efficient at hydrolyzing several peptides with hydrophobic residues at the P1 position. Moreover, the regulator stimulates cleavage of these same hydrophobic peptides to a greater extent by MCP containing the MHC II-encoded subunits. Since IFNγ induces the synthesis of LMP2, LMP7 and the 29 kDa regulator subunit, it seems likely that the cytokine alters the substrate specificity of MCP in vivo. In addition, the observed changes in specificity are consistent with the idea that MCP is responsible for generating peptides presented on class I molecules.

Table 2
Stimulation of MCP peptidase activities by RBC vs. recombinant regulator

	Stimulation (x-fold)					
	RBC-MCP		721.45 MCP		721.174 MCP	
	Red cell regulator	Recomb. regulator	Red cell regulator	Recomb. regulator	Red cell regulator	Recomb. regulator
Basic						
FSR-MCA	16	8	12	11	21	21
PFR-MCA	9	7	10	9	6	11
VLK-MCA	20	14	12	8	16	22
GKR-MCA	8	6	6	5	7	11
Acidic						
LLE-2NA	12	11	14	10	7	12
Hydrophobic						
LLVY-MCA	27	20	27	23	19	39
AAF-MCA	20	12	20	12	18	19
GGF-MCA	2	2	6	5	2	2
LY-MCA	11	8	16	15	10	11

Each entry presented above is an average from 2 to 6 independent experiments.

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