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Immunology

Effects of interferon γ and major histocompatibility complex-encoded subunits on peptidase activities of human multicatalytic proteases

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ABSTRACT **We have examined several peptidase activities of human multicatalytic protease (MCP) purified from the lymphoblastoid cell line 721.45 and a deletion mutant derivative, 721.174, lacking MCP subunits encoded in the major histocompatibility complex (MHC) class II region. Wild-type lymphoblast MCP hydrolyzed a specific peptide, glutaryl-Gly-Gly-Phe-4-methylcoumaryl-7-amide (-MCA), several times faster than the mutant enzyme did, suggesting that MHC-encoded subunits may provide this activity. Contrary to a recent report [Driscoll, J., Brown, M. G., Finley, D. & Monaco, J J. (1993) *Nature (London)* 365, 262–264], we did not detect significant aminopeptidase associated with lymphoblast MCPs. Our results also differ markedly from those of Gaczynska *et al.* [Gaczynska, M., Rock, K. L. & Goldberg, A L. (1993) *Nature (London)* 365, 264–267], who reported that γ interferon (IFN-γ) alters the peptidase activities of lymphoblast MCPs. We found that IFN-γ did not produce significant differences in the peptidase activities of purified MCPs. Moreover, our measurements of V_{max} and K_m for succinyl-Leu-Leu-Val-Tyr-MCA hydrolysis differ 600-fold and 15-fold, respectively, from those reported by Gaczynska *et al.* On balance, the findings presented here do not support the idea that IFN-γ induces major changes in the peptidase activity of purified MCPs.**

It is now well accepted that antigen presentation involves the production of peptides that bind major histocompatibility complex (MHC) class I or II receptors (1, 2). It is also clear that extracellular proteins are the source of most peptides bound to MHC class II molecules, and MHC class I molecules display peptides from intracellular proteins (either self- or pathogen-encoded). Presumably, endosomal proteases are responsible for producing the peptides bound to MHC class II molecules (3). The multicatalytic protease (MCP) or 20S proteasome is thought to generate the peptides displayed by MHC class I molecules (4). MCP is a cylinder-shaped particle that forms the proteolytic core of an even larger, 26S protease (5–7). Several pieces of evidence suggest MCP and the 26S protease generate peptides for class I receptors. First, two MCP subunits, low molecular mass polypeptides LMP2 and LMP7, are encoded in the MHC locus (8–10). Second, synthesis of LMP2 and LMP7 is induced by interferon γ (IFN-γ) (11), a cytokine that induces many cellular components involved in antigen presentation (12). Third, ubiquitin-mediated proteolysis has been implicated in antigen presentation (13), and MCP is a component of the 26S protease that degrades ubiquitin conjugates.

MHC class I molecules bind to peptides that are 8–10 residues long and generally possess hydrophobic or positively charged C termini (14). The restricted C termini of presented peptides suggested that LMP2 and LMP7 might cleave after hydrophobic or positive residues, thereby conferring MCP with the ability to generate peptides likely to bind MHC class I molecules (15). The hypothesis received a set-back when two groups found that LMP2 and LMP7 were not necessary for efficient antigen presentation (16, 17). On the other hand, two recent papers (18, 19) offer support for the "immunosubunit" hypothesis by reporting that IFN-γ alters the peptidase activities of MCP in a manner expected for efficient presentation. However, these papers contain observations inconsistent with the known properties of MCP. Accordingly, we attempted to confirm their principal findings, and we have obtained markedly different results.

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MATERIALS AND METHODS

Materials and Cell Lines. Fluorogenic peptides were purchased from Sigma, Enzyme Systems Products, or Peninsula Laboratories. Human IFN-γ was purchased from Chemicon. Human lymphoblastoid cell (LC) lines 721.45 and 721.174 were gifts from R. DeMars at the University of Wisconsin. Human erythrocyte MCP was prepared as described (20). LC were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum FCS in the presence or absence of 200 units of IFN-γ per ml for 6 days. MCPs were purified from 2×10^8 LC cells mixed in 2 ml of lysis buffer (0.25% Triton X-100/1 mM dithiothreitol/10 mM Tris, pH 7.6). The extract was clarified in a Microfuge for 10 min at 4°C, and the supernate was treated as described under *Results*. Protein was assayed by the method of Bradford (21). Peptidase activity was assayed either at room temperature or at 37°C as described (22). Native gel electrophoresis and fluorogenic peptide overlay were performed as described (22).

RESULTS

Peptidase Activities of Human Multicatalytic Proteases. Purified human erythrocyte MCP was incubated with each of three single-amino-acid-extended 4-methylcoumaryl-7-amide (MCA) substrates (Leu-MCA, Arg-MCA, and Asn-MCA) and with each of three standard fluorogenic peptides [succinyl-(Suc)-Leu-Leu-Val-Tyr-MCA, Pro-Phe-Arg-MCA, and Leu-Leu-Glu-2-naphthylamide (-βNA)]. Degradation of each substrate is shown in Table 1. After correction for spontaneous hydrolysis, it is evident that cleavage of Leu-MCA, Arg-MCA and Asn-MCA by the multicatalytic protease is less than 1.0% of that oberved for blocked peptides. This result is consistent with our previous finding that rabbit reticulocyte MCP has virtually no aminopeptidase activity (23).

However, hydrolysis of amino acid-MCA substrates was attributed to MCP obtained from human LC (18). This raises the possibility that aminopeptidase activity is present in MCPs

Abbreviations: IFN-γ, interferon γ; MHC, major histocompatibility complex; MCP, multicatalytic protease; -MCA, 4-methylcoumaryl-7-amide; LMP, low molecular mass polypeptide; LC, lymphoblastoid cells; Glt, glutaryl; Suc, succinyl; Cbz, benzyloxycarbonyl; -βNA, β-naphthylamide; Boc, butoxycarbonyl; wt, wild type.

Table 1. Peptidase activities of human erythrocyte MCP

	4-Methyl-7-aminocoumarin or β-naphthylamine generated, pmol							
	Buffer only			Buffer + MCP			Net pmol from MCP	
	0 h	1 h	2 h	0 h	1 h	2 h	1 h	2 h
L-MCA	7	13	19	7	13	20	<1	1
R-MCA	4	5	7	5	6	8	1	1
N-MCA	5	10	15	9	10	16	<1	1
sLLVY-MCA	—	0	—	—	1144	—	1144	—
PFR-MCA	—	0	—	—	346	—	346	—
LLE-βNA	—	0	—	—	1015	—	1015	—

Purified human erythrocyte MCP (3 μg) was incubated with 100 μM amino acid-MCA substrates at room temperature for the indicated times. Fluorogenic peptide assays were identical except that incubation was for 5 min, and the product formed was multiplied by 12 to obtain the Table entry. Single-letter amino acid code is used.

from lymphoblasts but not from erythroid cells. To determine whether the substrate specificity of MCP differs between the two cell types, we obtained wild-type (wt) lymphoblastoid cell line 721.45 and the deletion mutant 721.174. Since it also has been reported that IFN-γ affects the substrate specificity of lymphoblastoid MCPs (19), we assayed peptidase activities from LC grown in the presence or absence of IFN-γ.

Crude extracts of lymphoblasts were prepared and clarified as described above. Both resuspended pellets and supernates were assayed immediately with Suc-Leu-Leu-Val-Tyr-MCA, glutaryl(Glt)-Gly-Gly-Phe-MCA, and Asn-MCA. Although pellets were essentially devoid of peptidase activity, all three substrates were cleaved by enzymes in the supernate (see Table 2). Significant differences between wt and mutant LC extracts were particularly evident with Glt-Gly-Gly-Phe-MCA as substrate. The specific activity for hydrolysis of this peptide was almost 20-fold higher in extracts from wt cells. This difference can be attributed to MCP as demonstrated by peptide overlay after native gel electrophoresis (Fig. 1). Whereas Suc-Leu-Leu-Val-Tyr-MCA was cleaved to a similar extent by both wt and mutant cells, cleavage of Glt-Gly-Gly-Phe-MCA could barely be detected upon electrophoresis of crude extract from mutant cells. Mixing experiments produced no evidence for an activator of Glt-Gly-Gly-Phe-MCA hydrolysis in wt extract or for an inhibitor in mutant extract. These results suggest that LMP2 and/or LMP7 confer Glt-Gly-Gly-Phe-MCA hydrolytic activity to MCP.

Extracts from wt and mutant LC also differed 2-fold in their ability to cleave Suc-Leu-Leu-Val-Tyr-MCA (Table 2). However, in this case, there was clear indication of an activator in wt extract. That is, mixtures containing 1 part wt cell extract and 4 parts mutant cell extract hydrolyzed Suc-Leu-Leu-Val-Tyr-MCA at the same rate as wt extract (not shown). Results in Table 2 also reveal that treatment of either wild-type or mutant cells with IFN-γ did not produce statistically significant differences in their ability to cleave any of the substrates. In fact, rates of Asn-MCA hydrolysis were almost identical in all extracts.

Separation of Aminopeptidases from the MCP. To determine whether amino acid-MCA and peptide-MCA activities resided in the same enzyme, LC extracts were chromatographed on Toyopearl-DEAE-650S. When Suc-Leu-Leu-Val-Tyr-MCA and Asn-MCA were used as substrates, two overlapping but distinct peaks of peptidase activity were observed (Fig. 2). The enzyme that hydrolyzed Suc-Leu-Leu-Val-Tyr-MCA was eluted from DEAE at the salt concentration expected for MCP, and it was confirmed to be the MCP by its sedimentation coefficient of 20S on a 10–40% glycerol gradient; the Asn-MCA aminopeptidase, by contrast, sedimented at 11S (data not shown).

Substrate Specificities of Partially Purified LC Peptidases. To characterize the Asn-MCA aminopeptidase and to test for

Table 2. Effects of IFN-γ on peptidase activities in human LC extracts

LC cell type	IFN-γ	Specific activities, pmol/min per μg		
		sLLVY-MCA	gGGF-MCA	N-MCA
wt	−	21.1 ± 2.2*† (n = 4)	7.8 ± 1.3‡§ (n = 3)	0.5 ± 0.04¶ (n = 4)
wt	+	25.6 ± 3.7*† (n = 3)	11.4 ± 2.6‡§ (n = 2)	0.5 ± 0.06¶ (n = 4)
Mut	−	10.8 ± 1.7† (n = 3)	0.5 ± 0.1§ (n = 3)	0.4 ± 0.04¶ (n = 3)
Mut	+	12.0 ± 2.0† (n = 3)	0.6 ± 0.1§ (n = 2)	0.4 ± 0.02¶ (n = 3)

Human LC extracts (2 μl) were incubated with 100 mM fluorogenic substrates at 37°C for 20 min under the conditions specified in text. Statistical significance of the differences among specific activities was calculated by using a standard "t" test (24). LC lines: wt, 721.45; deletion mutant (Mut), 721.174. NS, not significant. Single-letter amino acid code is used.

*wt− vs. wt+, NS (P = 0.1).

†Mut− vs. Mut+, NS (P = 0.5). wt− vs. Mut−, significant (P < 0.01). wt+ vs. Mut+, significant (P < 0.01).

‡wt− vs. wt+, almost significant ($P \cong 0.1$).

§Mut− vs. Mut+, NS (P = 0.5). wt− vs. Mut−, significant (P = 0.01). wt+ vs. Mut+, significant (P < 0.05).

¶NS.

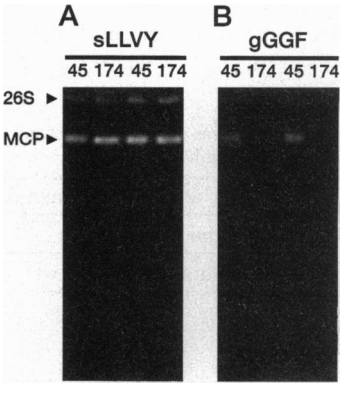

FIG. 1. Peptidase activities of wt 721.45 (lanes 45) and mutant 721.174 (lanes 174) LC MCPs assayed by native gel overlay. LC were extracted with 100 μl of lysis buffer per 10^7 cells. The extract was centrifuged for 10 min in a Microfuge at 4°C, and 20-μl aliquots of supernate were applied in duplicate to lanes of a native gel as described (22). After electrophoresis, the gels were overlaid with 100 μM Glt-Gly-Gly-Phe-MCA (*B*) or Suc-Leu-Leu-Val-Tyr-MCA (*A*) and incubated for 20 min at room temperature. Fluorescence was detected by transillumination with a UV lamp. 721.174 LC lack genes coding for LMP2 and LMP7.

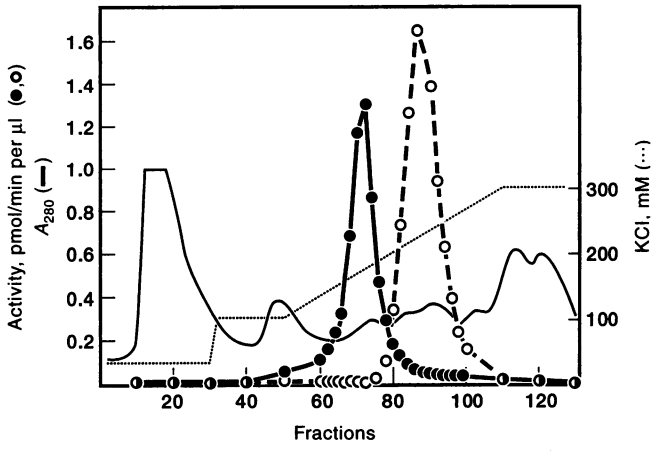

FIG. 2. Ion-exchange chromatography of LC peptidases. Cells (≈2 × 10^8) were mixed in 2 ml of lysis buffer, and the extract was clarified by centrifugation. The supernate was added to a 2-ml column of TSK-DEAE-650S equilibrated in 10 mM Tris·HCl, pH 7/25 mM KCl/10 mM NaCl/1.1 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol. The column was eluted with 10 ml of the same buffer containing 0.1 M KCl, followed by a 0.1–0.3 M KCl gradient. Fractions (0.5 ml) were collected and assayed for protein (absorbance at 280 min) and peptidase activity. ●, Asn-MCA hydrolysis; ○, Suc-Leu-Leu-Val-Tyr-MCA hydrolysis.

the presence of aminopeptidase activity in purified lymphoblast MCPs, we assayed peak fractions of MCP and Asn-MCA aminopeptidase obtained after glycerol gradient centrifugation with four blocked peptides and four amino acid-MCA derivatives. The data in Table 3 show that Asn-MCA aminopeptidase actually prefers Leu-MCA and may hydrolyze the tripeptide Pro-Phe-Arg-MCA to a minor extent. Based on its modest (6-fold) specificity for Leu-MCA and the fact that it is insensitive to bestatin (not shown), the Asn-MCA-hydrolyzing enzyme does not appear to be leucine aminopeptidase (25). The Asn-MCA peptidase sediments faster than a second aminopeptidase also present in DEAE fractions 65–75 (see Fig. 2) that prefers Leu-MCA 100-fold more than Asn-MCA (not shown). This smaller, bestatin-sensitive enzyme is likely to be leucine aminopeptidase. We did not characterize either aminopeptidase further.

Partially purified lymphoblast MCPs exhibited traces of aminopeptidase activity preferential for Leu-MCA (Table 3). However, in all cases, hydrolysis of Leu-MCA by LC MCPs was <10% of that observed with Suc-Leu-Leu-Val-Tyr-MCA, and

Table 3. Substrate specificities of lymphoblastoid Asn-MCA (N-MCA) aminopeptidase and MCPs

	Specific activity, pmol/min per μg							
	N-MCA aminopeptidase				MCP			
	wt		Mutant		wt		Mutant	
	−	+	−	+	−	+	−	+
sLLVY	−	−	−	−	44	36	58	48
GGF	−	−	−	−	14	12	6	5
PFR	1.3	−	2.8	1.7	5	4	4	4
LLE	−	−	0.2	0.1	8	6	23	16
L-MCA	91	98	206	164	2.5	2.9	1.0	1.7
R-MCA	41	45	95	85	1.7	2.2	0.5	1.5
F-MCA	13	14	33	23	0.4	0.5	0.3	1.1
N-MCA	16	17	34	28	−	−	0.1	0.1

Fluorogenic substrates (100 μM) were incubated with 200 ng of each enzyme in the presence (+) or absence (−) of IFN-γ for 30 min at 37°C. See text for assay conditions. Single-letter amino acid code is used. A dash in the table indicates the data were < 0.1 pmol/min per μg.

cleavage of Asn-MCA was 1/10th of that. Because Leu-MCA cleavage in purified MCP was inhibited 50% by bestatin (not shown), we attribute amino acid-MCA hydrolysis to minor contamination of isolated MCP by both leucine and "Asn-MCA" aminopeptidases. In any event, we found Asn-MCA cleavage by LC MCPs to be <2% of the Glt-Gly-Gly-Phe-MCA cleavage activity in contrast to Driscoll *et al.* (18), who reported 5 times more hydrolysis of Asn-MCA than Glt-Gly-Gly-Phe-MCA.

Relative hydrolysis of two blocked peptides, carbobenzoxy (Cbz)-Leu-Leu-Glu-βNA and Glt-Gly-Gly-Phe-MCA, differed between MCPs from wt and mutant cells. MCPs from mutant LC degraded Cbz-Leu-Leu-Glu-βNA severalfold faster than the enzyme from wt cells, and depending upon the preparation, the wt enzyme degraded Glt-Gly-Gly-Phe-MCA 3–6 times faster than the mutant enzyme.

Kinetics of Peptide Hydrolysis by MCPs from 721.45 and 721.174 Human LC. IFN-γ was reported to alter fluorogenic peptide hydrolysis by lymphoblast MCP (19). Because DEAE fractionation and glycerol gradient sedimentation produced reasonably pure enzymes, we attempted to confirm the reported effects of IFN-γ on the protease. LC were grown in the presence or absence of IFN-γ for 6 days, MCPs were purified by DEAE and glycerol gradient and then incubated with various concentrations of Suc-Leu-Leu-Val-Tyr-MCA, butoxycarbonyl(Boc)-Phe-Ser-Arg-MCA, or Cbz-Leu-Leu-Glu-βNA. Hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA followed Michaelis–Menten kinetics (Fig. 3*A*) with apparent K_m values of 30 μM or 20 μM and V_{max} values of 60 and 30 pmol/min per μg for wt and mutant cells, respectively. Quite different results were obtained with the peptides Phe-Ser-Arg-MCA and Cbz-Leu-Leu-Glu-βNA. As found with MCPs from other tissues (26, 27), hydrolysis of the blocked Arg-MCA peptide did not exhibit saturation, indicating that the K_m for this substrate is >500 μM (Fig. 3*B Upper*). And in agreement with three studies on hydrolysis of Cbz-Leu-Leu-Glu-βNA by MCP (28–30), we observed a biphasic relationship between enzyme velocity and the concentration of this fluorogenic peptide (Fig. 3*B Lower*). Although wt and mutant cells differed in the concentration-dependent hydrolysis of Cbz-Leu-Leu-Glu-βNA, IFN-γ treatment of the cells did not affect hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA, Pro-Phe-Arg-MCA, or Leu-Leu-Glu-βNA by purified MCPs from either cell line.

Electrophoretic Pattern of MCP from wt and Mutant Human LC. To assess the relative purity of MCPs isolated from mutant and wt LC, we analyzed glycerol gradient-purified enzymes by SDS/PAGE. Silver-stained SDS/polyacrylamide gels of MCP from 721.45 and 721.174 cells show clear differences in subunit pattern (Fig. 4). We consider it likely that the two "extra" bands present in wt MCPs correspond to the LMP2 and LMP7 subunits. Densitometry indicated that MCP subunits compose >60% of the total protein in the partially purified MCP fractions.

DISCUSSION

The experiments presented above were prompted by two recent reports that (*i*) attribute high levels of aminopeptidase activity to MCPs from lymphoblasts (18) and (*ii*) claim that IFN-γ affects the endopeptidase activities of the enzyme (19). Contrary to those publications and in agreement with earlier studies (23, 31, 32), we found virtually no aminopeptidase activity in erythrocyte MCP (Table 1) and only trace activity in lymphoblast MCPs (Table 3). Moreover, IFN-γ did not affect the endopeptidase activities of lymphoblast MCPs (Table 3 and Fig. 3).

Unable to confirm either finding, we now consider several possible explanations for the discrepancies between our results and those in refs. 18 and 19. We obtained the LC lines 721.45 and 721.174 from the same source as Driscoll *et al.* (18) and

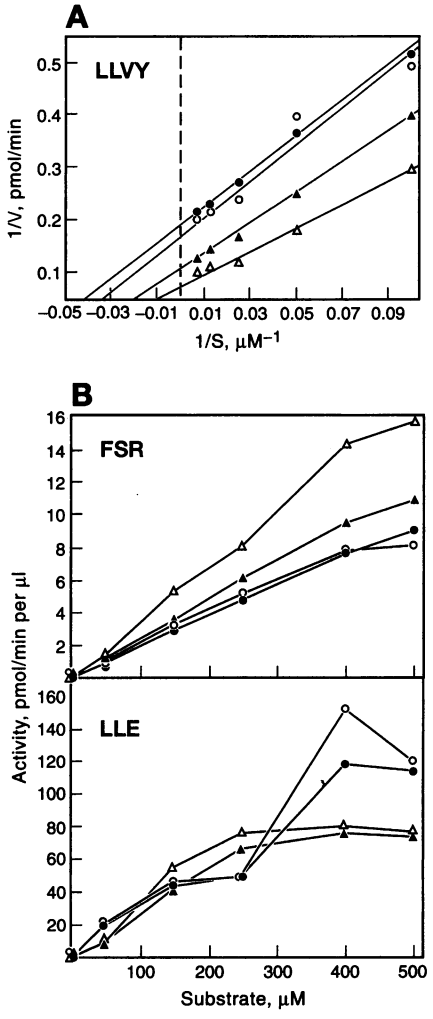

FIG. 3. Kinetics of fluorogenic peptide hydrolysis by wt and mutant lymphoblast MCPs. Kinetic data are presented in a Lineweaver–Burk plot for Suc-Leu-Leu-Val-Tyr-MCA (*A*) and as velocity versus substrate concentration for peptides Boc-Phe-Ser-Arg-MCA (*B Upper*) and Cbz-Leu-Leu-Glu-βNA (*B Lower*). MCP (210 ng per reaction) was incubated with increasing concentrations of each substrate in 30 mM Tris (pH 7.0)/0.5 mM dithiothreitol/5 mM $MgCl_2$/10 mM KCl; the final concentrations of glycerol and dimethyl sulfoxide were 24% and 5%, respectively. Initial velocities were obtained by incubation at 37°C for 5 and 15 min (*A*) or for 20 and 40 min (*B*). ● and ▲, with IFN-γ; ○ and △, without IFN-γ; △ and ▲, wt LC 721.45; ○ and ●, mutant LC 721.174.

Gaczynska *et al.* (19). Consequently, the observed experimental differences cannot be attributed to cell-type variation. Since the source of IFN-γ was Genzyme for the studies in ref. 18 and unreported in ref. 19, we cannot be certain of equivalence. However, IFN-γ stimulates synthesis of a protein (33) later shown by us to be an activator of MCP (34). IFN-γ from Chemicon induces synthesis of the MCP activator (34), indicating that the recombinant cytokine is active. Enzyme extraction procedures differ between our studies and the previous reports, but this seems unlikely to account for the conflicting results because MCP is a remarkably stable enzyme (5–7).

We assume that our findings differ from those of Driscoll *et al.* (18) because they performed their experiments with MCP heavily contaminated by (or associated with) aminopeptidases. In support of this assumption, we identified in LC extracts an aminopeptidase with activity against Asn-MCA and with chromatographic properties similar to MCP (Fig. 2). Since the Asn-MCA aminopeptidase sediments at 11S, it could contaminate MCP following the gel filtration step used by that group. We cannot explain, however, why Driscoll *et al.* (18) found Asn-MCA to be the preferred substrate.

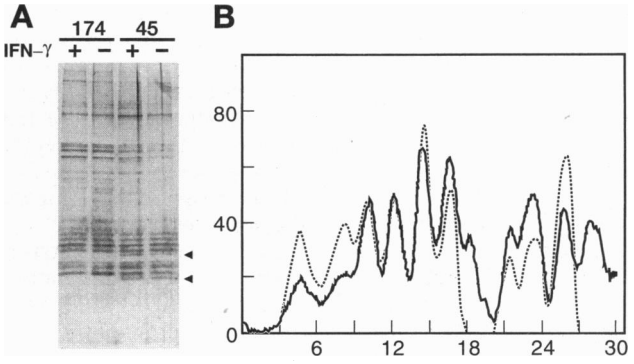

FIG. 4. SDS/PAGE profiles of mutant and wt lymphoblast MCPs. (*A*) Electrophoretic patterns of human MCP from wt LC line 721.45 (lanes 45) and mutant LC line 721.174 (lanes 174) with (lanes +) and without (lanes −) IFN-γ. Aliquots of purified MCP (500 ng) were dissolved in SDS sample buffer, boiled for 2 min, and applied to lanes of a SDS/10% PAGE gel. After electrophoresis at 200 V, proteins were detected by silver staining. Arrowheads indicate the subunits missing from mutant LC MCP. (*B*) Densitometric scans were carried out with a Joyce–Loebl Chromoscan 3. ———, 721.45; --------, 721.174.

We also believe that our results differ from those of Gaczynska *et al.* (19) because of errors in their kinetic analyses. Here we note that they often determined K_m values by using only a 2-fold range in substrate concentration. In addition, their Michaelis–Menten plots for hydrolysis of Leu-Arg-Arg-MCA (figure 2 in ref. 19) can be drawn through the origin, indicating the absence of saturation. And as shown in Table 4, their values for hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA diverge markedly from results obtained in three laboratories other than this one. We do agree that wt and mutant LC differ in their ability to cleave Cbz-Leu-Leu-Glu-βNA. However, the situation is complicated by the biphasic aspects of hydrolysis versus substrate concentration. For example, at 200 μM Cbz-Leu-Leu-Glu-βNA, wt MCP hydrolyzes the substrate better than does the mutant enzyme (Fig. 3*B*). Hence, the conclusion that wt MCP is deficient in this activity is too simplistic.

In contrast to both studies (18, 19), we were unable to confirm any effect of IFN-γ on the peptidase activities of purified MCPs (Fig. 3 and Table 3). Again, we attribute the differences to inaccurate measurements in the previous studies. Although our kinetic results differ markedly with those of Gaczynska *et al.* (19), they agree with the studies of Boes *et al.* (37) with one exception. Boes *et al.* (37) found that IFN-γ treatment of mouse fibroblasts resulted in a 60% inhibition of Suc-Leu-Leu-Val-Tyr-MCA hydrolysis by purified fibroblast

Table 4. Kinetic parameters for hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA by MCPs from various tissues

Source of MCP	K_m, μM	V_{max}, pmol/min per μg	Ref.
Bovine RBCs	200	50	35
Rat liver	150	—	36
Mouse fibroblasts	18	49	37
Human monocytes	0.0004	0.02	19
Human LC			
wt	500	0.11	19
Mut	300	0.04	19
wt	30	60	This work
Mut	20	30	This work

Mut, mutant; RBCs, erythrocytes.

MCP. In some preparations, we observed differences in apparent V_{max} values for Suc-Leu-Leu-Val-Tyr-MCA hydrolysis between wt and mutant cells (Fig. 3*A*), whereas in others we did not (Table 3). Hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA is very sensitive to the presence of an hexameric activator (35, 38). We suspect that incomplete removal of this MCP effector, differential contamination by other proteins, and possible enzyme inactivation during purification can account for the observed variation in final specific activities.

The data in Table 2 show a large difference in hydrolysis of Glt-Gly-Gly-Phe-MCA by crude extracts from wt and mutant LC. However, the activities obtained with purified MCPs were much closer (Table 3). The basis for this apparent discrepancy is still uncertain, but it appears to be due (unpublished data) to an ≈4-fold difference in substrate hydrolysis inherent to the MCPs coupled with an almost 3-fold greater response of wt MCP to a previously characterized activator of the MCP (35, 38). Since synthesis of the activator is induced by IFN-γ (33, 34), we propose that LMP2 and LMP7 may well bias substrate cleavage toward production of presentable peptides. However, in our view, they function by modest differences in their substrate specificities as revealed by certain fluorogenic peptides (e.g., Glt-Gly-Gly-Phe-MCA) coupled with enhanced response to the activator described in refs. 35 and 38.

In summary, we have detected consistent differences in the hydrolysis of Glt-Gly-Gly-Phe-MCA by MCPs from wt and mutant lymphoblastoid cells. However, we did not confirm that IFN-γ treatment of LC directly affects the peptidase activities of *purified* MCPs. Thus, our findings and those of Boes *et al.* (37) cast serious doubt on the reports that IFN-γ directly alters the enzymatic activity of MCP to provide presentable peptides for MHC I molecules. However, IFN-γ may influence the generation of peptides through effects on a potent activator of MCP (38).

Note Added in Proof. Since this paper was submitted for publication, Gaczynska *et al.* (19) have published a second set of kinetic constants for peptide hydrolysis by the multicatalytic protease from lymphoblast line 721.174 (39). For certain peptides, the results reported in ref. 39 differ by at least an order of magnitude from those reported in ref. 19 and are closer to the data reported in this manuscript.

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