ELSEVIER

FEBS Letters 343 (1994) 85–88

FEBS 13920

FEBS LETTERS

Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-γ for acquirement of the functional diversity responsible for antigen processing

Kinya Akiyama[a], Susumu Kagawa[a], Tomohiro Tamura[b], Naoki Shimbara[c], Makoto Takashina[c], Poul Kristensen[d], Klavs B. Hendil[d], Keiji Tanaka[b,*], Akira Ichihara[b]

[a]*Department of Urology, School of Medicine, and* [b]*Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan*
[c]*Biomaterial Research Institute, 1 Taya-cho, Sakae-ku, Yokohama 244, Japan*
[d]*August Krogh Institute, University of Copenhagen, 13 Universitetsparken, DK 2100 Copenhagen 0, Denmark*

Received 10 March 1994

Abstract

Proteasomes catalyze the non-lysosomal, ATP-dependent selective breakdown of ubiquitinated proteins and are thought to be responsible for MHC class I-restricted antigen presentation. Recently, we reported that gamma interferon (IFN-γ) induced not only marked synthesis of the MHC-encoded proteasome subunits LMP2 and LMP7, but also almost complete loss of two unidentified proteasome subunits tentatively designated as X and Y in various human cells. Here, we show that subunit X is a new proteasomal subunit highly homologous to LMP7, and that subunit Y is identical to the LMP2-related proteasomal subunit delta. Thus, IFN-γ appears to induce subunit replacements of X and Y by LMP7 and LMP2, respectively, producing 'immuno-proteasomes' with the functional diversity responsible for processing of endogenous antigens.

Key words: Antigen processing; MHC class I; Interferon-γ; Multicatalytic proteinase; Proteasome; Ubiquitin

1. Introduction

Recent progress in studies on the mechanism of the MHC class I-restricted antigen presentation pathway have indicated that intracellular antigenic peptides generated by the ubiquitin pathway are transported into the endoplasmic reticulum through the TAP1/2 heterodimeric peptide transporter [1,2]. Cytosolic proteasomes functioning as an extra-lysosomal, ATP-dependent protease to selectively degrade ubiquitinated proteins [3,4] are therefore proposed to be involved in the processing of intracellular antigens. Two novel proteasomal genes LMP2 and LMP7 were found to be localized within the MHC class II region, closely linked to the TAP1 and TAP2 genes [5]. Moreover, expression of these four polymorphic genes was found to be markedly up-regulated by gamma-interferon (IFN-γ), a major immunomodulatory cytokine [5,6], which is secreted by activated T-cells and NK cells in response to non-self antigens.

Recently, IFN-γ was found to change the specificity of proteasomes for peptide degradation, increasing their activities for endoproteolytic cleavage of peptide bonds on the carboxyl side of basic and neutral amino acid residues of proteins, but depressing their activity for peptides containing acidic amino acid residues [6–8]. This altered specificity is apparently consistent with the properties of antigenic peptides associated with cell surface class I molecules [9]. However, it is unknown how the specificity of proteasomes is changed, although an important role for the LMP2 and LMP7 subunits in the complex has been suggested [5]. We recently found that IFN-γ resulted in not only a high induction of LMP2 and LMP7 synthesis, but also a marked reduction in the synthesis of two newly-identified subunits of proteasomes, tentatively named X and Y [6]. In the present work, we report that these X and Y subunits are a novel class of proteasomal subunits with high homology to LMP7 and LMP2, respectively, and that subunit Y is identical to proteasomal subunit delta, as reported previously [10]. These findings suggest that the specificity of proteasomes is changed by an IFN-γ-induced change of subunits from X/Y to LMP7/2, and that this change may be responsible for accelerated processing of non-self endogenous antigens.

2. Materials and methods

The 20S proteasome from human kidney was purified to near homo-

*Corresponding author.

SSDI 0014-5793(94)00305-F

geneity by conventional chromatographic techniques, as described before [11]. Peptide antibodies were raised in rabbits by immunization with peptides corresponding to the C-terminals of LMP2 (HRVILGNELPKFYDE) and LMP7 (TDVSDLLHQYREANQ) as described [6]. A monoclonal antibody against the delta subunit of human proteasomes was produced in hybridoma cells ([12]; Hendil et al., in preparation). Immunoelectrophoretic blot analysis was carried out by the method of Towbin et al. [13]. Samples (50 μg protein) separated by two-dimensional electrophoresis by the method of O'Farrell (2D-PAGE) [14] were transferred electrophoretically to Immobilon-PVDF membranes (Millipore). The membranes were pretreated with Block Ace (Yukijirushi Co., Sapporo), and immunoreactive protein detected with antibodies and an ECL system (Amersham).

For the isolation of X and Y, samples (150 μg protein) of proteasomes were subjected to 2D-PAGE, transferred to membranes, and detected by staining with Coomassie brilliant blue. The spots corresponding to X and Y from approximately 35 membranes were excised and digested with lysyl-endopeptidase or trypsin. The digest was separated by reverse-phase HPLC on a μBONSADPHERW C18–100Å column (2.1 × 150 mm) developed with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The eluate was monitored by measuring the absorbance at 215 nm. The peak fractions collected were used for N-terminal amino acid sequencing. Peptides were subjected to automated Edman degradation in a Model 477A gas-phase sequencer (Applied Biosystems, Inc.), and phenylthiohydantoin derivatives were identified with a Model Applied Biosystems 120A phenylthiohydantoin amino acid analyzer on-line system.

3. Results

3.1. Identification of proteasome subunits X and Y down-regulated in response to IFN-γ

The exact subunit composition of the human 20S proteasome was examined by 2D-PAGE. The purified enzyme from human kidney was separated into multiple components with molecular masses (M_r) of 21 kDa to 33 kDa with a range of isoelectric points from 4 to 10 (Fig.1A). The protein profile was very similar to that of the fluorogram obtained for the immunoprecipitate with anti-proteasomal antibody from cell extracts metabolically labelled with [^{35}S]methionine reported recently [6]. The protein spots corresponding to LMP2 and LMP7 (shown by arrows) were detected by immunoblot analysis with specific antibodies against LMP2 and LMP7, which had been raised in rabbits by immunization with peptides synthesized on the basis of the C-termini of their primary structures reported before [6]. LMP2 and LMP7 were identified as newly-synthesized subunits that were markedly induced by treatment with IFN-γ [6]. On the other hand, the two spots designated as X and Y (indicated by circles) were found to be the two subunits down-regulated in response to IFN-γ detected in the fluorogram mentioned above, based on their Mrs and pIs and the positions of other proteasomal subunits [6]. To obtain information on the structures of these proteasomal subunits X and Y, we analyzed them by protein-chemical and immunological techniques.

3.2. Subunit Y is identical to proteasomal subunit delta with a high homology to LMP2

For determining the primary structure of protein Y, after transfer from 2D-PAGE to PVDF membranes, its spot was excised and digested with lysyl-endopeptidase. The digest was separated by reverse-phase HPLC and the peak fractions were used for N-terminal amino acid sequencing. One fragment of Y was determined to have the sequence FAVATLPPA, which was completely in

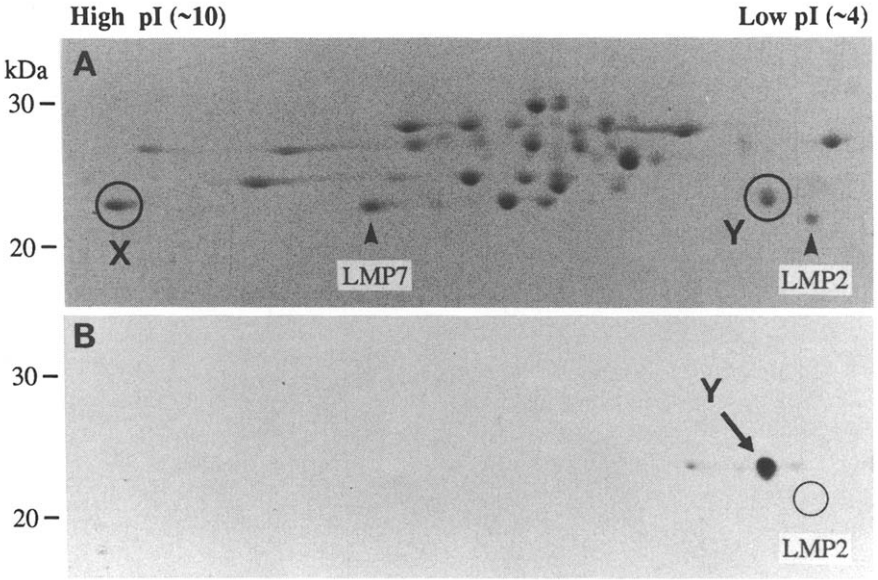

Fig. 1. Electrophoretic analysis of human kidney proteasomes and immunological identification of Y as subunit delta. (A) Subunit pattern of human kidney proteasomes separated by 2D-PAGE and stained with Coomassie brilliant blue. The two spots shown by arrowheads were identified as LMP2 and LMP7 by immunoblot analysis with their respective antibodies [6]. The two spots shown by circles were markedly down-regulated by IFN-γ treatment in various cell lines, and were tentatively named X and Y [6]. (B) Immunoblot analysis using anti-delta monoclonal antibody MCP421. The positions of Y and LMP2 are shown by an arrow and stippled circle, respectively. Note that anti-delta reacted specifically with the spot of Y, but not with that of LMP2.

Fig. 2. Partial structural analysis of subunit X from human liver proteasomes. Sequence alignment of peptides derived from subunit X with those of human LMP7. Identical amino acid residues are boxed by black. Subunit X was digested with lysyl-endopeptidase (peptides a and b) or trypsin (peptides c and d) and the fragments separated by reverse-phase HPLC.

accord with the C-terminal sequence of the proteasomal subunit delta, as judged by a computer-assisted homology research. Subunit delta has been reported by DeMartino et al. [10] to be a unique proteasomal subunit, showing high similarity with LMP2, although its cDNA clone did not encode the full-length translational region. Moreover, we found that anti-delta monoclonal antibody reacted specifically with the spot Y on immunoblotting analysis after separation by 2D-PAGE. This anti-delta antibody did not react with LMP2 (Fig.1B), despite the high sequence similarity of these two porteins (over 60% similarity). Thus we conclude that subunit Y is identical to delta and possibly related in function to LMP2

3.3. *Subunit X is a new proteasomal subunit highly homologous to LMP7*

To obtain the structural information on the protein named X, we generated several peptides by digestion with lysyl-endopeptidase or trypsin from the spots transferred to PVDF membranes after 2D-PAGE and isolated the resulting peptides by reversed-phase HPLC (data not shown). By N-terminal amino acid sequencing, the amino acid sequences of four fragments were determined to be as shown in Fig. 2A. Sequence alignment of the peptides with the primary structure of human LMP7 indicated that the peptides derived from protein spot X were very similar to those of LMP7 (Fig. 2). However, X was not identical to LMP7 itself, because anti-LMP7 antibody reacted specifically with subunit LMP7 of the proteasomal subunits, but did not react with X (data not shown). Moreover, the sequence TTXLAFK is almost identical to that of a peptide fragment named proteasomal subunit Epsilon (TTILAFKFRXGVIVAADSRATAGGYF) reported by Lee et al. [15]. The Epsilon sequence is highly similar, but not identical, to that of LMP7. Thus, we conclude that the X is a novel proteasomal subunit that is closely related with LMP7.

4. Discussion

In the present study, we demonstrated that proteasomal subunits X and Y, which are down-regulated in response to IFN-γ treatment, have extremely high similarity with class II MHC-encoded subunits LMP7 and LMP2, respectively, which are up-regulated by IFN-γ. These findings strongly suggest that IFN-γ induces substitutions of LMP7 and LMP2 for X and Y, forming proteasomes with a different subunit organization. Recently, we [6] and others [7,8] reported that INF-γ affects the proteolytic functions of proteasomes, greatly enhancing their chymotrypsin-like and trypsin-like peptidase activities and decreasing their peptidylglutamyl-peptide hydrolyzing activity. These changes of peptidase activities apparently correspond to the properties of peptides associated with cell surface class I molecules, because the carboxyl sides of these antigenic peptides consist mainly of hydrophobic and basic amino acid residues, not acidic amino acid residues (for review, see [9]). Thus alteration in the structural organization of proteasomes by IFN-γ modifies their functions, presumably to accelerate the processing of non-self antigens. These findings suggest that intracellular antigens can be processed by IFN-γ-modified proteasomes and that the peptides generated are transported into the endoplasmic

reticulum through the two putative transporters to be assembled with MHC class I molecules for presentation on the cell surface. Thus, IFN-γ-induced structural (and possibly functional) heterogeneity of subunit assembly of proteasomes may be responsible for immuno-regulations, such as antigen processing/presentation. Based on these findings, we propose the name 'immuno-proteasomes', for proteasomes containing LMP2 and LMP7 subunits, with functional diversity acquired through alteration of their subunit assembly in response to IFN-γ.

Previously, we reported that IFN-γ greatly increased the levels of the mRNAs encoding LMP2 and LMP7 without affecting those of any other subunits examined in various types of human cells, in which enhanced levels of these two mRNAs were roughly correlated with those detected by immunoblot analysis with their antibodies. This finding suggests that the IFN-γ induced proteasomes are formed by de novo synthesis, possibly due to change of transcription. Moreover, we found in pulse-chase experiments that the IFN-γ-dependent change of the subunit pattern resulted in alterations in the assembly of newly synthesized subunits, but not the reorganization or modification of pre-existing proteasomes, because IFN-γ did not affect the subunit pattern of pre-existing proteasomes [6]. Interestingly, however, the mRNA level of subunit delta (i.e. Y) was not affected by treatment with IFN-γ [6], suggesting that complete loss or a marked decrease in the expression of subunit Y and probably also X is due to regulation at the post-translational level. This alteration might be due to repression of their biosyntheses at the translational level. Alternatively, LMP2 and LMP7 may be assembled into the proteasomal complex preferentially to Y and probably also X, and the X and Y subunits not assembled into the complex may be degraded rapidly. The exact mechanism of subunit substitution is unknown, but it is of interest that the synthesis of X and Y is regulated at a post-translational level in response to IFN-γ, in marked contrast to the transcriptional regulations of the LMP2 and LMP7 genes. By using these novel mechanisms of action, IFN-γ induces the replacement of X/Y by LMP2/7, suggesting that proteasomes with different subunit composition are generated in cells in response to extracellular signals such as IFN-γ.

Molecular genetic studies on yeast have shown that most proteasome genes are essential for cell proliferation [3]. Interestingly, chromosomal deletion of the PRE2 gene, which is thought to be a homologue of LMP7 in budding yeast, is lethal [16]. In contrast, however, human cells lacking the LMP7 gene grow normally, indicating that this gene is dispensable [5]. This finding initially suggested that this subunit has different roles in different organisms. However, here we found that mammalian cells have two homologous genes encoding X with LMP7, and thus the actual homologue of PRE2 may be X, not LMP7, although it is unknown whether X is an essential gene. The evolution of the PRE2 gene seems interesting considering that it is essential in yeast and seems to be related to acquiring immunity in mammals.

Acknowledgements: This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan and the Novo Nordisk Foundation, Copenhagen.

References

[1] Goldberg, A. and Rock, K.L. (1992) Nature 357, 75–379.
[2] Michalek, M.T., Grant, E.P., Gramm, C., Goldberg, A.L. and Rock, K.L. (1993) Nature 363, 552–554.
[3] Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, A (1992) New Biol. 4, 173–187.
[4] Rivett, A.J. (1993) Biochem. J. 291, 1–10.
[5] Monaco, J.J. (1992) Immunol. Today 13, 173–179.
[6] Aki, M., Shimbara, N., Takashina, M., Akiyama, K., Kagawa, S., Tamura, T., Tanahashi, N., Yoshimura, T., Tanaka, K. and Ichihara, A. (1994) J. Biochem. 115, 257–269.
[7] Driscoll, J., Brown, M.G., Finley, D. and Monaco, J.J. (1993) Nature 365, 262–264.
[8] Gaczynska, M., Rock, K.L. and Goldberg, A.L. (1993) Nature 365, 264–267.
[9] Rammensee, H.-G., Falk, K. and Rötzschke, O. (1993) Annu. Rev. Immunol. 11, 213–244.
[10] DeMartino, G.N., Orth, K., McCullough, M.L., Lee, L.W., Munn, T.Z., Moomaw, C.R., Dawson, P.A. and Slaughter, C.A. (1991) Biochim. Biophys. Acta 1079, 29–38.
[11] Tanaka,K., Yoshimura,T., Kumatori,A., Ichihara,A., Ikai,A., Nishigai, M., Kameyama,K. and Takagi,T.(1988) J. Biol. Chem. 263, 16209–16217.
[12] Kaltoft, M.-B., Koch, C., Uerkvitz, W. and Hendil, K.B. (1992) Hybridoma 11, 507–517.
[13] Towbin, H.S., Staehelin, J. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[14] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[15] Lee, L.W., Moomaw, C.R., Orth, K., McGuire, M.J., DeMartino, G.N. and Slaughter, C.A. (1990) Biochim. Biophys. Acta 1037, 178–185.
[16] Heinemeyer, W., Gruhler, A., Mohrle, V., Mahe, Y. and Wolf, D.H. (1993) J. Biol. Chem. 268, 5115–5120.