RESEARCH COMMUNICATION

Association of immunoproteasomes with the endoplasmic reticulum

Paul BROOKS*, Rachael Z. MURRAY*, Grant G. F. MASON*, Klavs B. HENDIL† and A. Jennifer RIVETT*¹

*Department of Biochemistry, University of Bristol, School of Medical Sciences, Bristol BS8 1TD, U.K., and †August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100, Copenhagen O, Denmark

Proteasomes are complex multisubunit proteases which play a critical role in intracellular proteolysis. Immunoproteasomes, which contain three γ -interferon-inducible subunits, are a subset of proteasomes which have a specialized function in antigen processing for presentation by the MHC class I pathway. Two of the γ -interferon inducible subunits, LMP2 and LMP7, are encoded within the MHC class II region adjacent to the two TAP (transporter associated with antigen presentation) genes. We have investigated the localization of immunoproteasomes using monoclonal antibodies to LMP2 and LMP7. Immunoproteasomes were strongly enriched around the endoplasmic reticulum as judged by double-immunofluorescence experiments with anti-calreticulin antibodies, but were also present in the nucleus and throughout the cytosol. In contrast, proteasome subunit C2,

which is present in all proteasomes, was found to be evenly distributed throughout the cytoplasm and in the nucleus, as was the delta subunit, which is replaced by LMP2 in immunoproteasomes. γ -Interferon increased the level of immunoproteasomes, but had no effect on their distribution. Our results provide the first direct evidence that immunoproteasomes are strongly enriched at the endoplasmic reticulum, where they may be located close to the TAP transporter to provide efficient transport of peptides into the lumen of the endoplasmic reticulum for association with MHC class I molecules.

Key words: antigen processing, γ -interferon, LMP2, LMP7, MHC class I, proteasome.

INTRODUCTION

Proteasomes represent the major non-lysosomal proteolytic machinery of eukaryotic cells and are responsible for the degradation of many short-lived regulatory proteins, including cyclins and transcriptional regulators, and for some protein degradation associated with the endoplasmic reticulum (ER) [1,2]. They are also involved in the generation of peptides for antigen presentation by the MHC class I pathway [1,3].

20 S proteasomes have a cylindrical structure composed of a stack of four rings. In yeast there are seven different alpha and seven different beta type proteasome subunits with the outer alpha and inner beta rings each being made up of seven different subunits [4]. In mammalian cells there are ten different types of beta subunits, three of them (LMP2, LMP7 and MECL1) being non-essential subunits which are induced by γ -interferon. Two of the inducible subunits, LMP2 and LMP7, are encoded within the MHC class II region close to the TAP (transporter associated with antigen processing) genes which are required for transport of peptides into the lumen of the ER, where they bind to MHC class I molecules. Following treatment of cells with γ -interferon, LMP2, LMP7 and MECL1 are preferentially incorporated during the assembly of new proteasome complexes at the expense of three highly homologous subunits, delta (Y), MB1 (X) and Z [5,6]. These six subunits are catalytic subunits [7–9], and the incorporation of the γ -interferon-inducible subunits has been shown by several groups both to influence proteasome peptidase activities (see, e.g. [10]) and to increase the ability of cells to process and present antigens (see, e.g. [11,12]). Proteasomes containing the inducible subunits have been referred to as ‘immunoproteasomes’ [3].

The 20 S proteasomes form the catalytic core of larger proteasome complexes. The 26 S proteasome, which has 19 S regulatory complexes at each end of the core proteasome cylinder,

is responsible for ATP-dependent degradation of multi-ubiquitinated proteins [13] and some non-ubiquitinated proteins [14]. Another regulator protein, the 11 S regulator/PA28 or REG, can also bind at the ends of the 20 S proteasome cylinder and cause changes in peptidase activities. The 11 S regulator is induced by γ -interferon and is also known to facilitate antigen processing [15].

Results of many studies have demonstrated that proteasomes are present in the nucleus and throughout the cytoplasm of most mammalian cells (reviewed in [16]). We have previously studied the localization of 20 S proteasomes by immunogold electron microscopy using polyclonal anti-proteasome antibodies and found them to be in the nucleus, throughout the cytoplasm and closely associated to the ER [17]. Similar conclusions can be drawn from subcellular fractionation studies [18]. In order to look at the subcellular distribution specifically of the subpopulation of proteasomes (immunoproteasomes) involved in antigen processing, and to study the effect of γ -interferon on proteasome localization, we have raised monoclonal antibodies to the γ -interferon-inducible LMP2 and LMP7 subunits. We have chosen to use human embryo lung L132 cells, which normally express low, but easily detectable, levels of LMP2 and LMP7, and HeLa cells, which have negligible levels of these subunits prior to treatment with γ -interferon. The results from both cell types clearly demonstrate that, in contrast with the total proteasome population, immunoproteasomes are greatly enriched at the ER, and show a relatively low intensity of labelling in the cytoplasm and in the nucleus.

MATERIALS AND METHODS

Antibodies and immunoblotting

A mouse monoclonal antibody, MCP20 [19], which recognizes the C2 alpha-type proteasome subunit was used to investigate

Abbreviations used: TAP, transporter associated with antigen presentation; ER, endoplasmic reticulum; GFP, green fluorescent protein.

¹ To whom correspondence should be addressed (e-mail j.rivett@bristol.ac.uk).

the total proteasome population. MCP421 was used for detection of the delta subunit. Recombinant LMP2 and LMP7 proteins were produced from rat cDNAs [18] in *Escherichia coli* using the QIAexpressionist system (Qiagen, Crawley, West Sussex, U.K.) and used for the preparation of mouse monoclonal antibodies. The antibodies, Lmp2-13 and Lmp7-1 (both IgG1) were used for the present study. Immunoblotting was performed as described in [18] using the enhanced-chemiluminescence reagent from NEN or an alkaline phosphatase detection system as indicated in the Figure legends. Densitometric analysis of immunoblots was carried out using a Bio-Rad GS690 Imaging Densitometer with Molecular Analyst software. Purified rat liver 20 S proteasomes [20] were used for quantification. Anti-(C8 subunit) antibody (MCP72) was used instead of anti-(C2 subunit) antibody for total proteasomes because the anti-(C2 subunit) antibody does not recognize rat proteasomes.

Cell culture and preparation of extracts

Human embryo lung L132 cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with either 10% (v/v) newborn-bovine serum or 10% (v/v) fetal-bovine serum respectively, and penicillin (50 i.u./ml)/streptomycin (50 µg/ml) in a humidified CO₂/air (1:19) atmosphere. For immunofluorescence studies, subconfluent cultures were grown for 2 days in flasks in the presence or absence of 250 units/ml γ -interferon (Roche Molecular Biochemicals, Lewes, East Sussex, U.K.). Cells were then transferred to glass coverslips in 24-well plates (Nunc) at various cell densities and cultured for a further 24 h with or without γ -interferon.

Immunofluorescence microscopy

Immunofluorescence labelling was carried out as described previously [17,21], except that cells were fixed in methanol. Other fixing methods were tested but found to have little effect on the results obtained. Incubations with anti-LMP2 and anti-LMP7 antibodies were for 1 h at room temperature, followed by blocking for 30 min with rabbit serum and incubation for 1 h at 4 °C with FITC-conjugated rabbit anti-(mouse IgG) antibodies. For controls, the primary antibodies were omitted. Double-immunofluorescence experiments with rabbit polyclonal anti-calreticulin antibodies [22] were performed using Texas Red-conjugated goat anti-rabbit antibodies to detect anti-calreticulin antibodies, followed by FITC-conjugated rabbit anti-mouse antibodies (Dako Ltd., High Wycombe, Bucks., U.K.) to detect either anti-LMP2 or anti-LMP7 antibodies. Slides were examined using a Leica upright confocal microscope.

RESULTS

LMP2 and LMP7 subunit levels increase following treatment with γ -interferon

Immunoblots of cell extracts confirmed that the antibodies are specific for the proteasome subunits. In human embryo lung L132 cells the level of expression of LMP2 and LMP7 determined by immunoblot analysis increased up to 3–4-fold following treatment with γ -interferon (Figure 1). The greatest change in levels was observed after the first day of treatment, with only small further increases occurring after 2–3 days. A faint band corresponding to the precursor form of LMP2 and a barely detectable band of precursor LMP7 can be seen (in Figure 1A) after 1–2 days of γ -interferon treatment. The small amount of the precursor proteins relative to the mature forms of these subunits indicates that the precursor proteins are rapidly processed in L132 cells, presumably during incorporation into mature

Figure 1 Increase in levels of LMP2 and LMP7 following γ -interferon treatment of human embryo lung L132 cells and HeLa cells

(A) L132 cells were treated with 250 units/ml γ -interferon (IFN) for the number of days indicated in the Figure and then extracts (75 µg of protein) were run on SDS/PAGE gels for immunoblotting with anti-LMP2, anti-LMP7 and anti-C2 antibodies as indicated. Enhanced-chemiluminescence detection was used for LMP2 and LMP7, and alkaline phosphatase detection was used for C2. The position of precursor forms of LMP2 and LMP7 are indicated as pre-LMP2 and pre-LMP7, and bands labelled LMP2 and LMP7 are the mature processed subunits. (B) HeLa cells were grown for 3 days, either in the absence of γ -interferon or in the presence of γ -interferon, and then extracts were immunoblotted with anti-C2, anti-C8, anti-LMP2, anti-LMP7 and anti-delta antibodies as indicated.

proteasomes. The levels of proteasomes measured by immunoblotting with anti-C2 antibodies showed no significant change following γ -interferon treatment in either L-132 cells (Figure 1A) or HeLa cells (Figure 1B). 20 S proteasomes were measured in cell extracts at a level of 6.9 ± 0.8 µg/mg (mean $\pm$ S.D., $n = 3$) and approx. 60% were immunoproteasomes after treatment with γ -interferon. The decrease in the delta subunit following γ -interferon treatment was 38.3 ± 6.8 % (mean $\pm$ S.D., $n = 3$).

HeLa cells differ from L132 cells in that they express only very low levels of LMP2 and LMP7, if any, prior to γ -interferon treatment, after which they are easily detected (Figure 1B). After 3 days treatment with γ -interferon, delta decreased by 59.3 ± 4.5 % (mean $\pm$ S.D., $n = 3$). The decrease in delta subunit

Figure 2 Immunofluorescence labelling with anti-LMP2 and anti-LMP7 antibodies shows that immunoproteasomes are preferentially localized to the ER

Immunofluorescence labelling of human embryo lung L132 cells with anti-LMP2 antibodies before (A) and after (D) treatment for 3 days with γ -interferon and with anti-LMP7 antibodies before (G) and after (J) treatment with γ -interferon. (B) and (H) show immunofluorescence labelling of untreated HeLa cells with anti-LMP2 and anti-LMP7 antibodies respectively, which serve as a negative control, because HeLa cells do not express LMP2 and LMP7 in the absence of γ -interferon. (C) Shows the nuclear and cytoplasmic labelling of L132 cells with MCP20, a mouse monoclonal antibody recognizing proteasome alpha type subunit C2, which is present in all proteasomes. A similar distribution of total proteasomes is observed in HeLa cells (I). (D)–(F) show double-immunofluorescence labelling of the L132 cells which was carried out as described in the Materials and methods section using FITC-conjugated anti-LMP2 (green, D) with Texas Red-conjugated anti-calreticulin (red, E). (F) shows the superimposition of (D) and (E), with the yellow colours indicating co-localization. (J)–(L) show the double-immunofluorescence results obtained with anti-LMP7 (J) and anti-calreticulin (K) superimposed (L). Immunofluorescence labelling of LMP2 and LMP7 in γ -interferon-treated HeLa cells also showed a distribution similar to that of calreticulin (results not shown). In contrast, labelling of γ -interferon-treated L132 cells with anti-delta antibodies (M) and anti-calreticulin antibodies (N) gave a distinct pattern of immunofluorescence (M and N superimposed in O). In all cases controls obtained by omitting primary antibodies showed no labelling.

matched the increase in immunoproteasomes, so, following γ -interferon treatment, immunoproteasomes were present in HeLa cells at a similar level as in the induced L132 cells.

Proteasomes are present in the nucleus and throughout the cytoplasm, but immunoproteasomes are greatly enriched in the ER

Immunofluorescence labelling of subunit C2, which is present in all proteasomes, showed proteasomes to be present in the nucleus and in the cytoplasm in both L132 and HeLa cells (Figure 2C and 2I), as we and others have found previously. Although labelling was only weak in L132 cells which had not been treated with γ -interferon, the immunoproteasome-specific subunits, LMP2 and LMP7, were found to have a distribution significantly different from that of the total proteasome population (Figures 2A and 2G respectively). Only weak labelling was observed in the nucleus, and the labelling in the cytoplasm appeared to be associated with the ER. This distribution was clearer, but very similar, in cells which had been treated with γ -interferon (Figures 2D and 2J respectively), where the level of labelling was higher. Double-immunofluorescence studies were carried out with either anti-LMP2 or anti-LMP7 antibodies and antibodies against calreticulin, a major protein in the lumen of the ER. The results of the superimposition of labelling patterns for untreated cells (results not shown) and for γ -interferon-treated cells (Figures 2F and 2L) indicated a strong enrichment of both LMP2- and LMP7-containing proteasomes associated with the ER. Weak immunofluorescent labelling was detected throughout the cytoplasm and in the nucleus with both anti-LMP2 and anti-LMP7 antibodies.

The lack of immunofluorescent labelling with anti-LMP2 and anti-LMP7 antibodies in the HeLa cells prior to treatment with γ -interferon (Figures 2B and 2H) serves as a negative control and demonstrates that immunofluorescent labelling with these antibodies is specific for immunoproteasomes. Following treatment with γ -interferon, the enriched distribution of immunoproteasomes in HeLa cells also showed co-localization with the ER, as judged from the results of double-labelling experiments with Texas Red-conjugated anti-calreticulin antibodies (results not shown). In contrast with these results, labelling of both L132 cells and HeLa cells (interferon- γ -treated) with anti-delta antibodies appeared throughout the cytoplasm and nucleus (Figure 2M), similar to that obtained with C2 (see Figure 2C). The labelling with anti-delta showed a very distinct pattern from that with anti-calreticulin, but there was some co-localization (Figure 2O).

DISCUSSION

Several lines of evidence support the view that immunoproteasomes containing the MHC-encoded subunits LMP2 and LMP7 perform a specialized proteolytic function in antigen presentation by the MHC class I pathway (reviewed in [3]). Our studies on the localization of immunoproteasomes using monoclonal antibodies directed against the γ -interferon-inducible subunits LMP2 and LMP7 clearly demonstrate for the first time that this discrete subpopulation of proteasomes are distributed very differently from the total proteasome population and that they can be targeted to the ER membrane.

Immunofluorescence microscopy with monoclonal anti-C2 antibodies showed that proteasomes are present in the nucleus and in the cytoplasm, consistent with the results of previous studies using polyclonal antibodies [17,18,21]. By comparison, double-immunofluorescence studies using anti-LMP2 or anti-

LMP7 antibodies with anti-calreticulin antibodies showed that immunoproteasomes are strongly enriched at the ER membrane in the human embryo lung L132 cell line. There was apparently no change in the distribution of proteasomes following γ -interferon treatment, only an increase in their abundance, and there was no change in the amount of total proteasomes following γ -interferon treatment as reported by others using different cell types [10]. LMP2 was clearly strongly enriched at the ER both in L132 and in HeLa cells following treatment with γ -interferon, while the closely related delta subunit was distributed throughout the nucleus and cytoplasm. Although there is no obvious redistribution of total proteasomes after γ -interferon treatment, the results with anti-LMP2 and anti-LMP7 antibodies can be explained by a high enrichment of immunoproteasomes at the ER. The observed enrichment in both LMP2 and LMP7 at the ER may represent a relatively small proportion of the immunoproteasomes, because the ER only represents a very small proportion of the cell volume (2.5%; [17]). Immunoproteasomes were also detected in the nucleus and throughout the cytoplasm, but the labelling was much less intense.

A recent study [23] found green-fluorescent-protein (GFP)-tagged-LMP2-containing proteasomes to be distributed in the nucleus and cytoplasm in living cells. The apparent discrepancy between our results and the observation that GFP-tagged LMP2 is found in nuclear and cytosolic proteasomes in living cells [23] has several possible explanations based on the differences in the methods employed. Just how obvious the ER-associated labelling is may depend not only on the level of immunoproteasomes, but also on the level of detection. High levels of immunoproteasomes could saturate binding sites at the ER, and it is possible that there are some differences in their distribution in different cell types. The attachment of GFP to immunoproteasomes could also affect their distribution, possibly by blocking their normal association with the ER membrane. Interestingly, however, fluorescence recovery after photobleaching in the living cells [23] showed that the mobile fraction of immunoproteasomes in the cytosol was lower than that in the nucleus, which may also reflect an association of immunoproteasomes with the ER. Further evidence in support of this conclusion are the observations that LMP2 and LMP7 are enriched in microsomal fractions prepared from rat liver [18,24].

In addition to playing a catalytic role in antigen processing [15,16], proteasome subunits induced by γ -interferon clearly facilitate the interaction of proteasomes with the ER. Since the distribution of microsome-associated proteasomes on the outside of the smooth ER and the *cis*-Golgi [18] matches that of the TAP transporter [25], and since the LMP2 and LMP7 genes are closely linked to the TAP1 and TAP2 genes [3], it is conceivable that there may be a direct interaction between immunoproteasomes and TAP transporters located in the ER membrane. This would allow efficient transport of peptides from proteasomes into the lumen of the ER, where they become associated with MHC class I molecules. Interestingly the 11 S proteasome regulator (PA28), which is also induced by γ -interferon, is present predominantly in the cytosol, but does not show the enrichment around the ER [24]. Results of recent studies suggest that PA28 may play a key role in immunoproteasome assembly [26].

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