Primary structures of two homologous subunits of PA28, a γ-interferon-inducible protein activator of the 20S proteasome

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Abstract The primary structures of two proteins that comprise PA28, an activator of the 20S proteasome, have been determined by cDNA cloning and sequencing. These protein subunits, termed PA28α and PA28β, are about 50% identical to one another and are highly conserved between rat and human. PA28α and PA28β are homologous to a previously described protein, Ki antigen, whose function is unknown. PA28α, but neither PA28β nor Ki antigen, contains a 'KEKE motif', which has been postulated to promote the binding of proteins having this structural feature. PA28α and PA28β were coordinately regulated by γ-interferon, which greatly induced mRNA levels of both proteins in cultured cells. The mRNA level of the Ki antigen also increased in response to γ-interferon treatment, but the magnitude of the increase was less than that for the PA28s, and the effect was transient. These results demonstrate the existence of a new protein family, at least two of whose members are involved in proteasome activation. They also provide the basis for future structure/function studies of PA28 subunits and the determination of their relative physiological roles in the regulation of proteasome activity.

Key words: cDNA cloning; Multicatalytic proteinase complex; Proteasome; Proteasome activator PA28; γ-Interferon; Ki antigen

1. Introduction

The proteasome is a major intracellular protease involved in non-lysosomal mechanisms of protein degradation, including the constitutive bulk turnover of cellular proteins [1], the conditional degradation of specific proteins during temporally controlled processes such as the cell cycle [2], and the processing of antigens for presentation by class I major histocompatibility complexes [1,3]. Proteasome activity is mediated by specific regulatory proteins that form proteasome–regulator complexes [4]. One such regulator is a large multisubunit protein called PA700 [5–8]. PA700 mediates proteasome function in the ubiquitin-dependent proteolytic pathway. Proteins degraded by this pathway must first be modified by covalent conjugation to a polyubiquitin chain, which appears to target the substrate to the proteasome–PA700 complex by binding to at least one PA700 subunit [9]. Degradation of the substrate protein by the proteasome–PA700 complex occurs by an ATP-dependent mechanism whose biochemical basis is unclear. Although the proteasome's best-defined role is in the ubiquitin-dependent proteolytic pathway, not all proteasome regulators activate the degradation of ubiquitinated proteins. For example, another activator, termed PA28, is a 200,000-dalton protein composed of six 28,000-dalton subunits arranged in a ring-shaped structure [10–12]. This structure binds to one or both of the proteasome's terminal rings to form a proteasome–PA28 complex [12]. The proteasome–PA28 complex is activated with respect to the hydrolysis of small synthetic peptide substrates, but not with respect to the degradation of large proteins, regardless of their conjugation to polyubiquitin chains [10,11]. Unlike PA700's role in the regulation of the proteasome in the well-characterized ubiquitin-dependent proteolytic pathway [4], PA28's physiological role is unknown.

PA28 consists of at least two protein subunits of very similar molecular weight [11,13]. These subunits, designated in the current report as α and β, represent distinct but homologous gene products [13]. Rechsteiner and colleagues recently reported the primary structure for one of these proteins and showed that its cellular content was increased by treatment of the cells with γ-interferon [14]. Because γ-interferon also alters the expression of several proteasome subunits, resulting in changes in proteasome composition and possibly in the protease's kinetic properties, these findings suggest that the regulation of PA28 levels is part of an integrated response of the proteasome system to a common signalling pathway [15–18]. In order to learn more about the structure, function, and physiological role of PA28, it is essential to determine the primary structure of each of its constituent subunits. We report here the complete primary structures of the α and β subunits from rat and the β subunit from human. Furthermore, we show that the mRNA of each subunit is induced in human cells by γ-interferon.

2. Materials and methods

2.1. Construction of cDNA libraries

Total RNA was extracted from the poly(A)$^+$ RNAs of rat hepatoma H4TG cells and human hepatoblastoma HepG2 cells by the guanidin-

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI Nucleotide Sequence Databases with the following accession numbers: D45249 (rPA28α), D45250 (rPA28β) and D45248 (hPA28β).

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ium thiocyanate method [19]. Poly(A)$^+$ RNA was isolated by oligo(dT)30-latex (Oligotex-dT30; Takara Shuzu). For construction of cDNA libraries derived from H4TG and HepG2 cells, cDNAs were synthesized with a cDNA synthesis kit (Pharmacia LKB Biotechnology Inc.) using oligo(dT)$_{15}$ as a primer for synthesis of the first strand of cDNAs. The cDNAs were ligated with an *Eco*RI adapter containing a *Not*I site and then inserted at the *Eco*RI site of the λZAPII vector (Stratagene) for construction of the phage library.

2.2. Cloning and sequencing of cDNAs

To isolate a cDNA clone for rat PA28α, a H4TG cell cDNA library was screened by utilizing 727 bp of a ^{32}P-labeled PA28α cDNA previously isolated from a bovine brain library (Willy and DeMartino, unpublished data). Conditions of hybridization were described previously [20]. Twenty positive phages were isolated from about 5×10^5 recombinants, and the cloned cDNAs were excised in vivo as a pBluescript form by using a helper phage, R408. One of the longest clones had an insert size of approximately 0.9 kb and was further characterized by nucleotide sequencing using the dideoxy chain termination method using an automatic DNA sequencer (Pharmacia LKB Biotechnology Inc.).

For isolation of rat and human PA28β cDNA clones H4TG and HepG2 cell cDNA libraries in λZAPII were screened using a DNA probe generated by bovine liver cDNA templates and PCR-primers (5′-CGCATAAGGA(T/C)GA(T/C)GA(A/G)ATGGA-3′ and 5′-ACTGCCTCGTCTC(G/T)CTCATGTAGIAGIGC-3′). These oligonucleotide sequences were based on the published partial amino acid sequence of the bovine PA28β (27.3 kDa PA28) (245). The sense primer corresponded to the amino acid residues PPKDDEME, whereas the antisense primer corresponded to the amino acid residues ALVHERDEAV. The reaction was carried out under the conditions as described [20], with 30 cycles of denaturation (2 min at 94°C), annealing (2 min at 50°C), and polymerization (2 min at 72°C). The amplified products were cloned into pBluescript, and sequenced by the dideoxy chain termination method. The cloned fragment containing PA28 was excised from the plasmid, labeled with [α-^{32}P]dCTP, and employed as a probe for the plaque hybridization screening of cDNA libraries in λZAPII.

Nine positive phages were isolated from about 5×10^5 recombinants from the rat H4TG cell cDNA library by screening as described above. One of these clones with the longest insert was further characterized by sequencing. For isolation of human PA28β cDNA clones, about 5×10^5 plaques from the human HepG2 cDNA library were screened, and nine positive phages were isolated. Three of these clones were further characterized by sequencing. These clones, however, were incomplete in the 5′ untranslated and amino-terminal regions. For isolation of the 5′-terminal region of the cDNA, the first strand cDNA, primed with a specific primer, was amplified using the adapter primer [21] and another specific primer (the complement to nucleotides 321–339) (see Fig. 1C).

2.3. RNA blot analysis

For RNA blot analysis, human renal carcinoma KPK-1 cells were cultured as described previously [22]. γ-Interferon was added to culture medium at a final concentration of 500 U/ml, and culture was continued for 12, 24 and 48 h. Samples of 5 μg of total RNA from the cells were separated in agarose gels containing formaldehyde, transferred to nylon filters (Hybond N+, Amersham), and hybridized with cDNA probes labeled with [α-^{32}P]dCTP as described previously [23]. The probes used were a rat PA28α cDNA fragment (nucleotides 102–510), a PA28β cDNA fragment (nucleotides 243–591) generated by PCR using bovine liver cDNA templates, and a human Ki antigen cDNA fragment (nucleotides 469–990) kindly provided by Dr. Y. Nishida of Nagoya University [24]. β-Actin cDNA was obtained from Oncor.

3. Results

The proteasome activator, PA28, consists of at least two distinct but homologous protein subunits with molecular weights of approximately 28,000 [13]. In order to further define the structural relationships between these subunits, termed PA28α and PA28β, we determined their complete primary structures by cDNA cloning and sequencing. Using strategies described under section 2, a clone for each subunit was identified and sequenced from a rat cDNA library. A clone for PA28β was also identified and sequenced from a human cDNA library. The nucleotide sequence for each of these three cDNA clones and its respective deduced protein is shown in Fig. 1. Each sequence contained the entire coding region as well as 5′-and 3′non-coding regions. The 3′-non-coding region of each clone contained a putative polyadenylation signal (AATAAA), which was located 12, 13 and 19 nucleotides upstream from the poly(A) sequence for rat PA28α (rPA28α), rat PA28β (rPA28β), and human PA28β (hPA28β), respectively. We concluded that the ATG sequence assigned nucleotide numbers 1–3 in each clone is the respective initiation codon because it is preceded by a sequence that is similar to the consensus sequence for translation initiation [25], and because this assignment provides the longest open reading frame for a protein whose deduced structure has a molecular weight in good agreement with that of purified PA28. Comparison of the deduced sequences with amino acid sequences determined by automated Edman degradation of peptides of two bovine PA28 subunits previously reported by Mott et al. [13] indicated that the bovine subunit of mass 28,606 daltons is homologous to PA28α, whereas the bovine subunit of mass 27,290 is homologous to PA28β. Sequences of peptides from bovine PA28α were 93% identical (57 of 61 residues) to the corresponding deduced sequence of rat PA28α; sequences of peptides from bovine PA28β were 87% (101 of 116 residues) and 95% (110 of 116 residues) identical to the corresponding deduced sequences of rat and human PA28β, respectively (Fig. 1).

The rPA28α corresponds to a protein of 249 amino acids (M_r = 8,576) and its sequence is 93% identical to the sequence of a 249 amino acid PA28 protein deduced from a human cDNA clone recently reported by Rechsteiner and colleagues [14] (Fig. 2, Tables 1 and 2). The deduced PA28β proteins from rat and human contained 238 (M_r = 26,791) and 239 (M_r = 27,072) amino acids, respectively (Table 1). The rPA28β and hPA28β proteins were 89% identical to one another (Fig. 2, Table 2). These results indicate that each protein has been highly conserved between rats and humans. The hPA28α and hPA28β proteins were 47% identical to one another, and the rPA28α and rPA28β proteins were 48% identical to one another (Table 2).

The hPA28α is homologous to a previously described human protein, the Ki antigen [24] (Fig. 3). We have not identified the Ki antigen protein in our preparations of PA28, and it is unknown whether the Ki antigen protein plays any role in the regulation of proteasome function. In any case, the sequence of the Ki antigen is between 33% and 41% identical to the various PA28 sequences (Table 2).

The primary structures of PA28α subunits from rat and human (Figs. 2 and 3), as well as that from bovine PA28α (data not shown), contain a 'KEKE motif', as described by Realini et al. [14]. These regions, from amino acids 70–97, are characterized by a high content of alternating lysine and glutamic acid residues, and by other criteria established for this motif [26]. In contrast, neither the PA28β subunits nor the Ki antigen protein, contains this motif and, interestingly, this region of the aligned primary structures is one of the most dissimilar among the three proteins (Fig. 3).

Previous work showed that the level of PA28α protein was greatly increased by treatment of human cells with γ-interferon [14,27]. In order to determine whether PA28β and Ki antigen are also regulated by γ-interferon, KPK-1 cells were treated

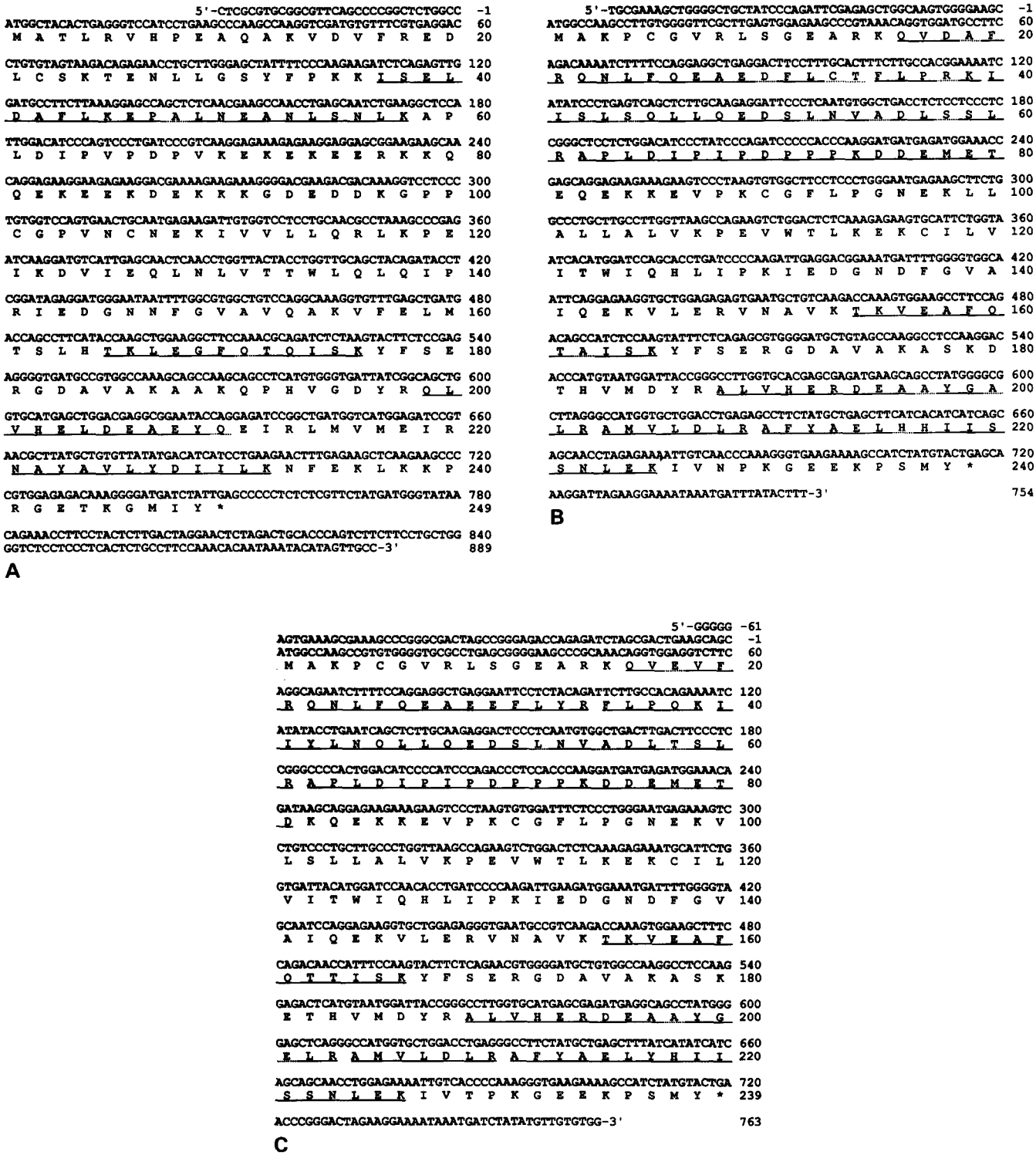

Fig. 1. (A) The nucleotide and deduced amino acid sequences of rat PA28α. Nucleotides are numbered starting from A of the translational initiation codon ATG. The asterisk (*) shows the termination codon. Continuous underlines show the amino acid sequences corresponding to those obtained by Edman degradation of peptides of purified bovine PA28α [13]. The amino acids shown by dotted lines were not identical with those found by chemical analysis of bovine PA28α. (B) The nucleotide and deduced amino acid sequences of rat PA28β. (C) The nucleotide and deduced amino acid sequences of human PA28β. The cDNA clone isolated from the cDNA library contained nucleotide positions 271–763. The 5'-terminal region was determined by the 5'-RACE.

with γ-interferon. After varied times of treatment, mRNA levels for each protein were determined by Northern blot analysis. The mRNA levels of both PA28α and PA28β were greatly increased within 12 h and remained elevated for the duration of the 48 h exposure of the cells to γ-interferon (Fig. 4). The mRNA level of the Ki antigen was also induced but by a lesser

Fig. 2. Alignment of amino acid sequences of PA28α from rat and human (A), and PA28β from rat and human (B). Solid lines indicate identity between the sequences.

magnitude than those for the PA28s. Furthermore, the Ki antigen mRNA level decreased toward control levels during the 48 h exposure to γ-interferon. These results indicate that the two PA28 proteins were regulated similarly in response to γ-interferon.

4. Discussion

We have determined the primary structures for two protein subunits, α and β, of PA28, an activator of the 20S proteasome. Together with a previous report [14], these data allow for the comparison of the primary structures of each subunit in two species, rat and human. As shown in Table 2, the primary structures of the α and β subunits are 47% and 48% identical to each other within each species, whereas the α subunits 93% identical and the β subunits are 89% identical between species. These data indicate that the divergence of the genes for PA28α and PA28β arose by duplication of a common ancestral gene before the rat and human species diverged. PA28α and PA28β are 33–41% identical to a third human protein, Ki antigen, previously identified as a nuclear protein detected with autoantibodies in sera of patients with systemic lupus erythematosus [24]. The cellular function of the Ki antigen is unknown and we have not detected this protein in our preparations of PA28.

Rechsteiner and colleagues have expressed PA28α cDNA in

Table 1
Properties of members of the protein family comprised of PA28α, PA28β, and Ki antigen

Human	AAs	M_r	pI	Rat	AAs	M_r	pI
hPA28α	249	28,741	5.71	rPA28α	249	28,576	5.70
hPA28β	239	27,072	5.33	rPA28β	238	26,791	5.28
hKi antigen	254	29,508	5.61				

The indicated properties of PA28α, PA28β, and Ki antigen were calculated from deduced amino acid sequences. The sequences for hPA28α and hKi antigen were taken from [14] and [24], respectively.

Table 2
Similarities among primary structures of PA28α, PA28β, and Ki antigen

	% of amino acid sequence identity				
	hPA28α	hPA28β	hKi antigen	rPA28α	rPA28β
hPA28α	100				
hPA28β	47	100			
hKi antigen	40	33	100		
rPA28α	93	48	41	100	
rPA28β	47	89	34	48	100

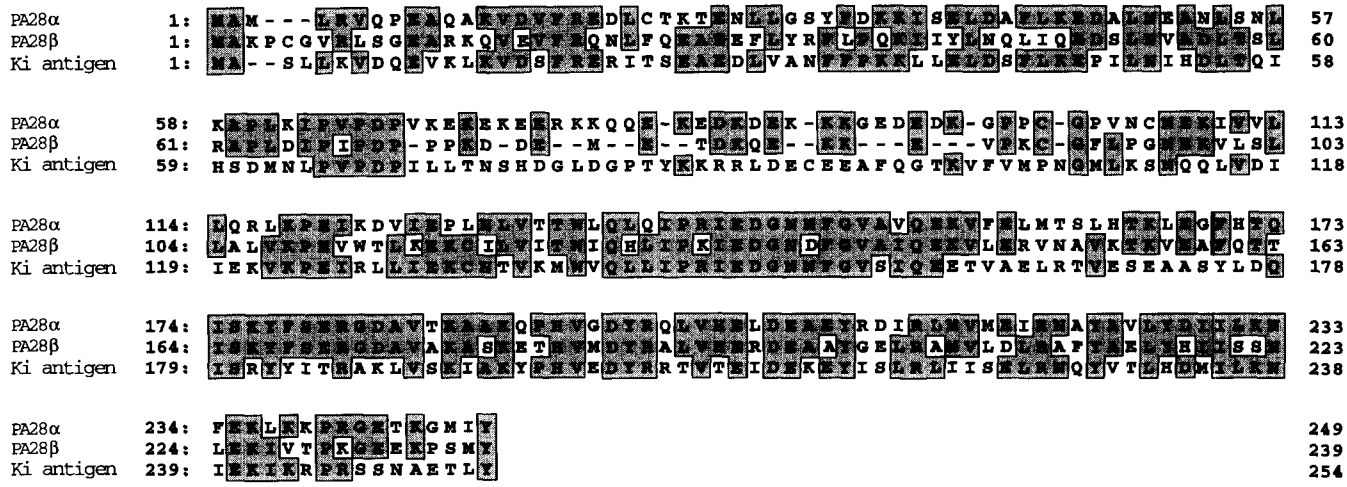

Fig. 3. Alignment of amino acid sequences of human PA28α, PA28β, and Ki antigen. The sequences of human PA28α and Ki antigen have been taken from published reports [14] and [24], respectively.

E. coli [14]. The resulting protein activated the proteasome similarly to native PA28 purified from cells. Thus, only one of the two PA28 subunits is sufficient for proteasome activation. Current biochemical data, however, have not clarified whether native PA28 is composed of multimers containing both α and β subunits (e.g. an $\alpha_3\beta_3$ structure), or whether the native protein consists of two distinct homohexamers (i.e. separate α_6 and β_6 proteins). Furthermore, it is unclear whether PA28 must form hexamers to activate the proteasome or whether monomers can also achieve this activity.

Despite an amino acid sequence that is similar to those of PA28α and PA28β, the Ki antigen protein appears to differ from PA28 in a number of respects. First, because the Ki antigen protein has not been identified in purified preparations of PA28 it may be separated from PA28 during purification, possibly because it differs from PA28 in any of the physical and/or biochemical properties used as the basis for PA28 purification. Analysis of the distribution of the Ki antigen protein with specific antibodies during PA28 purification should determine the relative abundance and fractionation of this protein when compared to PA28. Second, the Northern blot analysis of these proteins showed that the size of the mRNA for the α and β PA28s are very similar (0.9 kb), whereas the size of the mRNA for the Ki antigen is much larger (2.5 kb). Third, the mRNA levels for these three proteins responded differently to γ-interferon treatment (Fig. 4). Functional data regarding the ability of the Ki antigen to interact with and activate the proteasome will provide important insights into the relative structure-function relationships of these three proteins.

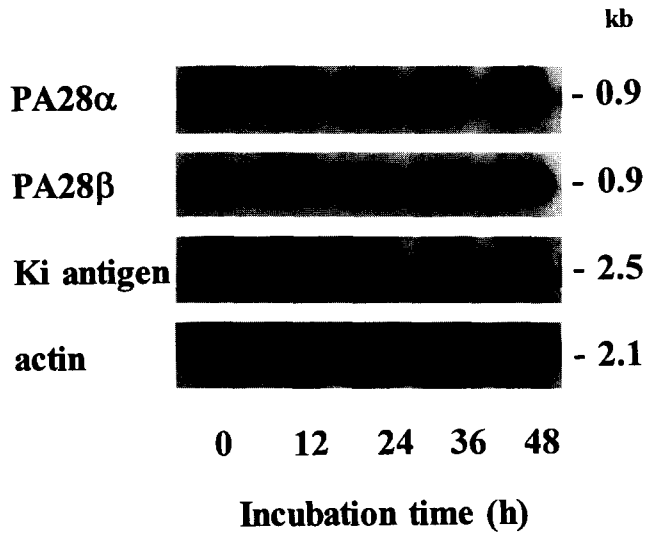

Fig. 4. RNA blot analysis from KPK-1 cells after treatment with γ-interferon. Samples of total RNA from KPK-1 cells were analyzed with each cDNA probe labeled with ^{32}P as described in section 2. The signals generated with the β-actin probe served as a control.

The mRNA levels of both PA28α and PA28β were greatly induced by treatment of cells with γ-interferon. These results extend two previous reports which showed that similar treatment increased cellular protein levels of PA28α [14,27]. This latter effect was originally described by Honoré et al. for a protein they designated IGUP I-5111 [27]. These workers also deduced the primary structure of the γ-interferon-inducible IGUP I-5111, but were unaware of its function as a proteasome activator. Subsequent cloning and sequencing of hPA28α established that the two proteins were identical [14]. Although the physiological significance of PA28 induction by γ-interferon is unclear, it is interesting to note that γ-interferon also regulates the subunit composition of the proteasome. Thus, γ-interferon up-regulates the expression of two proteasome subunits, LMP2 and LMP7 (both of which are encoded in the major histocompatibility complex), and reciprocally down-regulates two other proteasome subunits, X and Y (delta) [15–18]. The resulting alteration in proteasome composition has been correlated with altered proteasome function including modified kinetic properties and substrate specificity [17,28]. These results suggest that γ-interferon may promote a coordinated response of the proteasome system for a specific physiological functions, such as the proteasome's proposed role in the production of antigenic peptides for presentation by HLA class I molecules. Other work has also implicated the proteasome in antigen processing because of its role in the ubiquitin-dependent proteolytic pathway [1,29]. Although PA28 does not activate the proteasome's degradation of ubiquitinated proteins, it is unclear if it might

play a role in this function when associated with other proteins [5]. Alternatively, PA28 may mediate distinct ubiquitin-independent functions of the proteasome. The primary structures of both PA28 subunits and the γ-interferon regulation of PA28 reported here will provide the bases for additional studies to determine the physiological role of this activator.

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References

[1] Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and Goldberg, A.L. (1994) Cell 78, 761–771.
[2] Ciechanover, A. (1994) Cell 79, 13–21.
[3] Goldberg, A.L. and Rock, K.L. (1992) Nature 357, 375–379.
[4] Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993) J. Biol. Chem. 268, 6065–6068.
[5] Ma, C.-P., Vu, J.H., Proske, R.J., Slaughter, C.A. and DeMartino, G.N. (1994) J. Biol. Chem. 269, 3539–3547.
[6] Hoffman, L., Pratt, G. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22362–22368.
[7] Udvardy, A. (1993) J. Biol. Chem. 268, 9055–9062.
[8] Peters, J.-M., Franke, W.W. and Kleinschmidt, J.A. (1994) J. Biol. Chem. 269, 7709–7718.
[9] Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061.
[10] Ma, C.-P, Slaughter, C.A. and DeMartino, G.N. (1992) J. Biol. Chem. 267, 10515–10523.
[11] Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22369–22377.
[12] Gray, C.W., Slaughter, C.A. and DeMartino, G.N. (1994) J Mol Biol 236, 7–15.
[13] Mott, J.D., Pramanik, B.C., Moomaw, C.R., Afendis, S.J., DeMartino, G.N. and Slaughter, C.A. (1994) J. Biol. Chem. 269, 31466–31471.
[14] Realini, C., Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 20727–20732.
[15] Akiyama, K., Yokota, K., Kagawa, S., Shimbara, N., Tamura, T., Akioka, H., Nothwang, H.G., Noda, C., Tanaka, K. and Ichihara, A. (1994) Science 265, 1231–1234.
[16] Yang, Y., Waters, J.B., Früh, K. and Peterson, P.A. (1992) Proc. Natl. Acad. Sci. USA 89, 4928–4932.
[17] Boes, B., Hengel, H., Ruppert, T., Multhaup, G., Koszinowski, U.H. and Kloetzel, P.-M. (1994) J. Exp. Med. 179, 901–909.
[18] Belich, M.P., Glynne, R.J., Senger, G., Sheer, D. and Trowsdale, J. (1994) Curr. Biol. 4, 769–776.
[19] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[20] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Mannual, 2d edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
[21] Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[22] Kanayama, H., Tanaka, K., Aki, M., Kagawa, S., Miyaji, H., Satoh, M., Okada, F., Sato, S., Shimbara, N. and Ichihara, A. (1991) Cancer Res. 51, 6677–6685.
[23] Tanaka, K., Fujiwara, T., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S. and Nakanishi, S. (1990) Biochemistry 29, 3777–3785.
[24] Nikaido, T., Shimada, K., Shibata, M., Hata, M., Sakamoto, M., Takasaki, Y., Sato, C., Takahashi, T. and Nishida, Y. (1990) Clin. Exp. Immunol. 79, 209–214.
[25] Kozak, M. (1989) J. Cell Biol. 108, 229–241.
[26] Realini, C., Rogers, S.W. and Rechsteiner, M. (1994) FEBS Lett. 348, 109–113.
[27] Honoré, B., Leffers, H., Madsen, P. and Celis, J.E. (1993) Eur. J. Biochem. 218, 421–430.
[28] Gaczynska, M., Rock, K.L. and Goldberg, A.L. (1993) Nature 365, 264–267.
[29] Michalek, M.T., Grant, E.P., Gramm, C., Goldberg, A.L. and Rock, K.L. (1993) Nature 363, 552–554.