Molecular cloning and expression of subunit 12: a non-MCP and non-ATPase subunit of the 26 S protease

Wolfgang Dubiel[a,*], Katherine Ferrell[b], Renate Dumdey[a], Sybille Standera[a], Siegfried Prehn[a], Martin Rechsteiner[b]

[a]*Institut für Biochemie, Universitätsklinikum Charité, Humboldt-Universität, Hessische Str. 3–4, 10115 Berlin, Germany*
[b]*Department of Biochemistry, University of Utah, School of Medicine, Salt Lake City, UT 84132, USA*

Received 26 February 1995

Abstract **A cDNA encoding subunit 12 (S12) of human erythrocyte 26 S protease has been isolated, sequenced and expressed. The cDNA contains an open reading frame that encodes a 36.6 kDA protein 96% identical to mouse Mov-34 and 67% identical to its *Drosophila melanogaster* homolog. Based on the high degree of sequence identity between human S12, mouse and *Drosophila* Mov-34 proteins, we conclude that the Mov-34 gene product is a component of the 26 S protease. Antibodies produced against two S12 fragments, Met[1]–Tyr[95] ($S12_{f95}$) and Met[1]–Leu[205] ($S12_{f205}$), react with S12 transferred to nitrocellulose from SDS-PAGE. In contrast, after transfer from native gels, the epitope(s) recognized by anti-$S12_{f205}$ is exposed in the regulatory complex but appears to be masked when the regulatory complex associates with the multicatalytic protease.**

Key words: Human 26 S protease; Regulatory complex; Subunit 12; Mov-34

1. Introduction

The 26 S protease is resposible for degrading ubiquitin (Ub) conjugates [1]. The enzyme consists of about 30 different subunits. Most polypeptides with molecular masses between 20 kDa and 32 kDa are components of its proteolytic core, the multicatalytic protease (MCP) or proteasome. MCP associates with a regulatory complex in an ATP dependent reaction to form the 26 S protease. The regulatory complex is composed of at least 15 different subunits with molecular masses ranging from 25 to 110 kDa (for review see [2]).

Whereas the sequences of most of the MCP subunits have been determined (for review see [3]), the sequence and function of components in the regulatory complex are less well characterized. Identification of subunits 4, 6 and 7 of the 26 S regulatory complex as members of a novel family of ATPases suggests that one function of the regulatory complex is to move polypeptide substrates to the protease active sites [4–6]. S4, S6 and S7 together with SUG1 and TBP1 form a highly conserved subfamily of S4-like proteins, and all S4-like ATPases were predicted to be components of the 26 S protease [4]. Recently it has been shown that SUG1 is a subunit of yeast 26 S protease [7] and that it is a component of the regulatory complex from bovine red blood cells [8]. Thus, five components of the regulatory complex are likely to be ATPases.

Based on their sequences, at least 6 other subunits in the regulatory complex do not appear to be ATPases. These components are presumably involved in substrate selection or in maintaining the structural integrity of the complex. Here we present the primary structure of a non-ATPase subunit of the 26 S regulatory complex and show by immunoblotting that its N-terminus is masked upon formation of the native 26 S enzyme.

*Corresponding author.

2. Materials and methods

The 26 S protease was prepared and peptide sequence analysis was performed as described [4]. Western blots of non-denatured enzyme were performed with enzyme purified on a 10–40% glycerol gradient. Subunits of the regulatory complex are numbered as published [6]. Human S12cDNA was isolated using PCR from a pair of degenerate oligonucleotide primers corresponding to the ends of the S12 N-terminal peptide. A 5′-end labelled oligonucleotide probe derived from the PCR product was used to screen a human liver and a HeLa cDNA library in pEX1 vectors by standard methods [9]. The DNA from four positive clones was sequenced (US Biochemical Corp.). The longest clone from the HeLa library was 1042 bp.

Antibodies against S12 fragments were obtained by using the pQE vector expression system providing a His-tag at the N-terminus of the proteins (Qiagen). DNA corresponding to the specified fragments was obtained by PCR and subcloned into the pQE vectors using restriction sites internal to the amplification primers. The first fragment ($S12_{f95}$) encodes the initial 95 amino acids of S12 (Met[1] to Tyr[95]) and the expressed polypeptide of the second fragment ($S12_{f205}$) encompasses Met[1] to Leu[205]. The PCR products were verified by sequencing. The His-tagged translation products were purified on Ni-NTA resin (Qiagen) and antibodies against $S12_{f95}$ and $S12_{f205}$ were raised in sheep by standard methods. Western blots were performed with antibodies affinity purified against the His-tagged antigens at 500 ng/ml using the ECL Western blotting technique (Amersham). Analysis of the Western blots was made using the Molecular Analyst 2.0 program for the Bio-Rad Imaging Densitometer GS 670. Sequence alignments were made with the aid of the FASTA program [10].

3. Results and discussion

N-Terminal sequence analysis of S12 of the human erythrocyte 26 S protease revealed a free N-terminus for S12, and further Edman degradation produced a 28 amino acid sequence. With a synthetic oligonucleotide derived from the intrapeptide PCR product, we isolated a 1042 bp long cDNA from a HeLa cDNA library (see section 2). The cDNA revealed an open reading frame (ORF) starting from [79]ATG. This methionine codon is surrounded by a reasonable consensus sequence for eukaryotic translation initiation [11]. The ORF encodes a 321 amino acid polypeptide with a calculated molecular mass of 36.6 kDa which is very similar to the 36 kDa estimated for S12 by SDS-PAGE (Dubiel et al., Mol. Biol. Rep., in press)

As shown in Fig. 1, the sequenced S12 peptide matches the

SSDI 0014-5793(95)00288-X

Fig. 1. Amino acid sequence of subunit 12 from human 26 S protease. The sequence deduced from the isolated S12 cDNA is shown in the one-letter amino acid code. The N-terminal peptide sequenced directly is shown in white on black.

N-terminus of the expected translation product except for the initial methionine. Comparison of the putative translation product of S12 cDNA with proteins in current databases revealed 96% amino acid identity with the gene product of mouse Mov-34 [12] and 67% identity with a homolog from *Drosophila* [13]. Alignment of the human S12 sequence, the gene product of mouse Mov-34 (accession # M64641) and the Mov-34 protein from *Drosophila* (accession # M64643) is presented in Fig. 2. Considering the extensive similarities in sequence among the three proteins, we conclude that S12 is the human homolog of Mov-34. The excellent alignment between the human and mouse sequences evident in Fig. 2 strongly suggests that we have isolated the entire S12 cDNA even though the cDNA of the human ORF does not contain a stop codon.

Mov-34 protein/S12 is not homologous to any other known protein. Except for potential phosphorylation sites no obvious motifs currently summarized in the PROSITE program are found in the protein sequence. The only striking feature of the S12 amino acid sequence is a very hydrophilic region at its C-terminus with 'alternating' glutamates and lysines. This domain is called a KEKE motif and has been proposed to function as a region of protein–protein interaction [14].

To demonstrate that the putative S12 cDNA encodes a bona fide component of the 26 S regulatory complex we produced antibodies against the recombinant protein. As potential antigens, we chose regions of S12 having the lowest homologies with proteins in current databases. The fragments $S12_{f95}$ and $S12_{f205}$ and the appropriate antibodies were obtained as described in section 2. Fig. 3A shows immunoblots against SDS-PAGE separated subunits of the 26 S protease. Both anti-$S12_{f95}$ and anti-$S12_{f205}$ specifically reacted with S12 of the 26 S complex. Whereas anti-$S12_{f95}$ reacted with the $S12_{f205}$ fragment, there is proportionately far less cross-reactivity of anti-$S12_{f205}$ with $S12_{f95}$. Thus, the major epitope(s) of $S12_{f205}$ must be C-terminal to Tyr^{95}.

We also performed immunoblots against the 26 S protease, regulatory complex and MCP separated under non-denaturing conditions (Fig. 3B). It is clear that anti-$S12_{f95}$ and anti-$S12_{f205}$ react much better with the regulatory complex than with the assembled 26 S protease. Visual inspection indicates that there are equivalent amounts of 26 S and regulatory complex in lanes 5 and 7, respectively. Thus the absence of reactivity with the 26 S protease does not appear due to differences in protein concentration. This was verified by imaging densitometry. The ratio of antibody to protein signals for the regulatory complexes seen in lanes 6 and 7 are at least three times that of the 26 S protease. We conclude that the major epitopes recognized by both antibodies to S12 are masked in the assembled 26 S complex. Comparing lane 6 of anti-$S12_{f95}$ to that for anti-$S12_{f205}$, the stronger anti-$S12_{f205}$ signal suggests that a major portion of S12 exposed in the regulatory complex is C-terminal to Tyr^{95}. There is slight cross-reactivity with a species in lane 6 that appears to be the fast migrating form of MCP [15]. Since anti-$S12_{f205}$ does not react with purified 20 S on native gels (data not shown) or in SDS-PAGE (Fig. 3A), the signal could be due to association of some S12 subunits with MCP after disassembly of the 26 S protease. In this regard, it has been suggested that S12 may be involved in coupling the regulatory complex to MCP [14]. However, anti-$S12_{f205}$ is probably reacting with a faster migrating form of the regulatory complex termed RC* [16]. This complex, which lacks subunits 2 and 4, migrates very similarly to MCP.

Our results demonstrate that subunit 12 of the 26 S protease is identical with the gene product of Mov-34 and that the protein is highly conserved during evolution. A human homolog of Mov-34 has been mapped to chromosome region 16q23-q24. Disruption of the mouse gene by proviral integration leads to a recessive embryonic lethal mutation. It is interesting to note that embryos homozygous for the proviral integration develop normally in vitro to the blastocyst stage, but die shortly after uterine implantation [13]. Conditional lethality obtained by point mutations of other subunits of the 26 S regulatory complex in yeast, such as mts2 (the homolog of human S4) [17] and CIM5 (the homolog of human S7) [7] support the idea that the 26 S protease is essential for cell division. The ts mutants of mts2 and CIM5 are defective in anaphase chromosome separation at restrictive temperature. Why a few cell cycles are completed in mouse embryos with a disrupted S12 gene is not clear at the moment. There might be enough 26 S protease in the oocyte to suffice until the blastocyst stage. On the other hand, S12 may only become essential later in development.

Antibodies against S12 fragments do not react with any other subunit in the 26 S complex, indicating that S12 is a unique

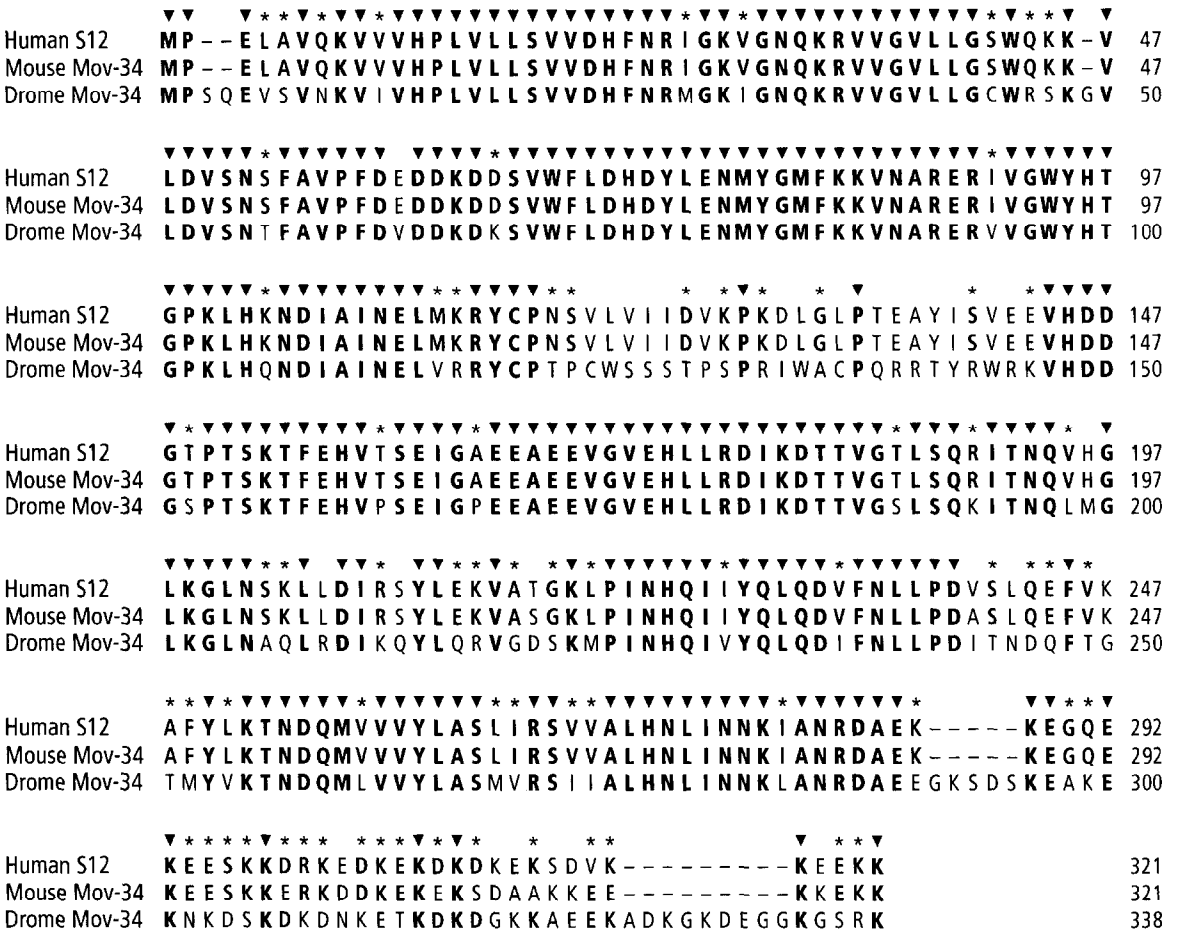

Fig. 2. Alignment of subunit 12 (Human S12) and the gene products of Mov-34 from mouse (Mouse Mov-34) and *Drosophila melanogaster* (Drome Mov-34). The amino acid sequences were aligned with the aid of the FASTA program. Identical amino acids (▼) and those conserved during evolution (*) are indicated.

component of the enzyme. The binding characteristics of anti-$S12_{f95}$ and anti-$S12_{f205}$ to native regulatory complex and 26 S protease suggests: (i) the N-terminus of S12 interacts with other components of the regulatory complex and is therefore masked in the assembled complex; (ii) some portion of the sequence between Tyr^{95} and Leu^{205} is exposed in the regulatory complex and masked when the regulatory complex binds MCP to form the 26 S protease.

In summary, we present here the primary structure of a non-proteasomal and non-ATPase subunit of the enzyme. Presently, 6 non-ATPase subunits in the regulatory complex have been identified (for rev. see Dubiel et al., Mol. Biol. Rep., in press). Each is a unique protein having no significant homologies with other polypeptides in current databases. Their functions are still unknown with the exception of S5a which confers polyubiquitin binding activity [17]. We speculate that the various non-ATPase subunits serve to target specific proteins for degradation, provide structural integrity to the regulatory complex or promote association of the regulatory complex with the multicatalytic protease.

Acknowledgements: These studies were supported by National Institutes of Health Grant GM37009 to M.R. and by a grant from Deutsche Forschungsgemeinschaft to W.D. (Du229/2-1) and by a grant from the Lucille P. Markey Charitable Trust.

References

[1] Hershko, A. and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807.
[2] Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993) J. Biol. Chem. 268, 6065–6068.
[3] Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, A. (1992) New Biol. 4, 173–187.
[4] Dubiel, W., Ferrell, K. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22699–22702.
[5] Dubiel, W., Ferrell, K. and Rechsteiner, M. (1994) Biol. Chem. Hoppe-Seyler 375, 237–240.
[6] Dubiel, W., Ferrell, K. and Rechsteiner, M. (1993) FEBS Lett. 323, 276–278.
[7] Ghislain, M., Udvardy, A. and Mann, C. (1993) Nature 366, 358–361
[8] DeMartino, G., Moomaw, C.R., Zagnitko, O.P., Proske, R.J., Ma, C.P., Afendis, S.J., Swaffield, J.C. and Slaughter, C.A. (1994) J. Biol. Chem. 269, 20878–20884.
[9] Ausubel, F. M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1992) Current Protocols in Molecular Biology, Green Wiley-Interscience, New York.
[10] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.

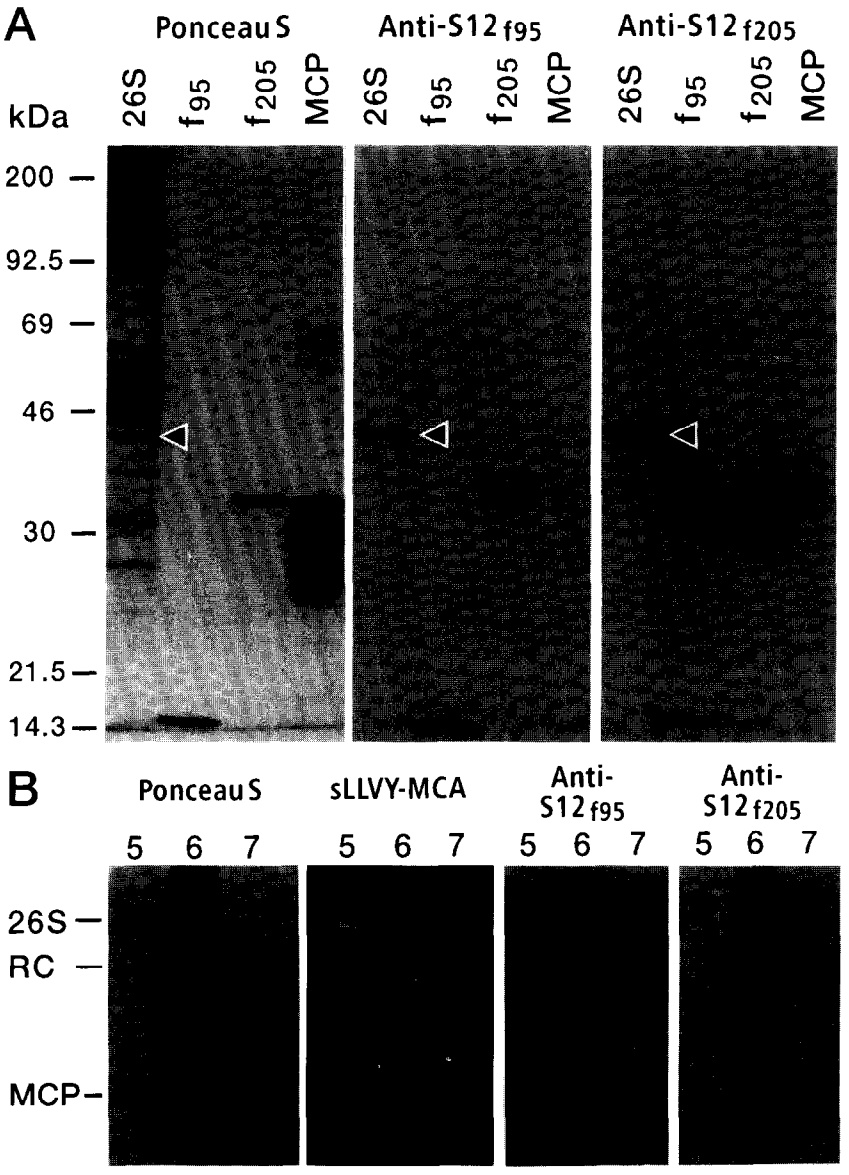

Fig. 3. Immunoblotting with anti-$S12_{f95}$ and anti-$S12_{f205}$. (A) 26 S, $S12_{f95}$, $S12_{f205}$ and MCP were separated by 12% SDS-PAGE (see section 2). Ponceau S stain of nitrocellulose (Ponceau S), immunoblotting with anti-$S12_{f95}$ (anti-f95) and immunoblotting with anti-$S12_{f205}$ (anti-f205). The arrow indicates the position of subunit 12. (B) The 26 S protease was separated into regulatory complex (RC) and the multicatalytic protease (MCP) on a glycerol gradient. Fractions 5, 6 and 7 were analyzed by non-denaturing electrophoresis. Ponceau S (Ponceau S) stain of nitrocellulose, overlay with the fluorescent substrate suc-Leu-Leu-Val-Tyr-MCA (sLLVY-MCA), immunoblotting with anti-$S12_{f95}$ (Anti-$S12_{f95}$) and immunoblotting with anti-$S12_{f205}$ (Anti-$S12_{f205}$).

[11] Kozak, M. (1986) Cell 44, 283–292.
[12] Gridley, T., Jaenisch, R. and Gendron-Maguire, M. (1991) Genomics 11, 501–507.
[13] Gridley, T., Gray, D.A., Orr-Weaver, T., Soriano, P., Barton, D.E., Francke, U. and Jaenisch, R. (1990) Development 109, 235–242.
[14] Realini, C., Rogers, S.W. and Rechsteiner, M. (1994) FEBS Lett. 348, 109–113.
[15] Hoffman, L., Pratt, G. and Rechsteiner, M. (1992) J. Biol Chem 267, 22362–22368
[16] Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061.
[17] Gordon, C., McGurk, G., Dillon, P., Rosen, C. and Hastie, N.D. (1993) Nature 366, 355–357.