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The Unfolding Story of the Chaperonins

ANTHONY R. M. COATES[1]*, BRIAN HENDERSON[2] AND PAOLO MASCAGNI[3]

[1]*Department of Medical Microbiology, St George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, UK,* [2]*Cellular Microbiology Research Group, Division of Surgical Sciences, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London, WC1X 8LD, UK and* [3]*Italfarmaco Research Centre, Via Lavoratori, 54, Cinisello Balsamo 20092, Milan, Italy*

Introduction

The chaperonins (cpn) are a family of sequence-related oligomeric proteins found in all cells which are essential for cell viability, particularly under conditions of environmental stress (Bakau, 1993; Minowanda and Welch, 1995). The genes encoding these proteins were discovered as part of the unfolding story of how cells respond to stress and the proteins themselves are now best known for their involvement in the non-covalent folding of proteins intracellularly (Ellis, 1996). As this article will delineate, the cpns also have biological actions in addition to those related to protein folding (Coates, 1996; Henderson *et al.*, 1996; Coates and Henderson, 1998; Lewthwaite *et al.*, 1998). The cpn family has two subfamilies, GroE and TCP1 (Coates *et al.*, 1993). The sequence identity within each subfamily is high, about 50%, but there is only around 20% identity between the subfamilies.

This review focuses on recent scientific advances in the GroE subfamily which contains two major proteins, cpn 10 and cpn 60 (Gupta, 1996); also known as GroES and GroEL (in *Escherichia coli*) (Georgopoulos *et al.*, 1972), heat shock protein 10 and 58/60/65 (McMullin and Hallberg, 1988; Gupta, 1995), 10/60/65 kDa antigen (Shinnick *et al.*, 1988; Baird *et al.*, 1989; Hoffman *et al.*, 1990), early pregnancy factor (Cavanagh, 1996) and P1 (Jindal *et al.*, 1989). The structural and folding aspects of the cpns have recently been comprehensively reviewed (Bukau and Horwich, 1998) and this article will concentrate on the non-folding functions of these proteins.

Gene organisation

In prokaryotes, the cpn 10 and cpn 60 genes are often located close to one another usually, but not always, in an operon (van der Vies and Georgopoulos, 1996). Many

*To whom correspondence may be addressed.

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organisms contain multiple cpn genes with related sequences, some of which are in operons while others are situated far from one another (Coates *et al.*, 1993). It is not clear what function these non-operon genes might fulfill. In eukaryotes, multiple cpn gene copies are also found (Gupta, 1996), and in the rat the cpn 60 and cpn 10 genes are linked head-to head, in a 14 kb assembly with 14 introns and a shared bidirectional promoter (Ryan *et al.*, 1997). So, whilst the gene arrangement in eukaryotes is different to that in prokaryotes, there are similarities, such as shared promoter regions.

Protein structure and folding properties

THE CHAPERONIN FOLDING MACHINE

Chaperonins belong to a group of proteins called molecular chaperones (Ellis, 1996) which bind non-native proteins and assist them, in an ATP-dependent catalytic process, to fold into the correct three-dimensional form required for a functional protein. The GroE chaperonin folding machine (Bukau and Horwich, 1998) is a large complex of about one million Daltons and assists many different proteins to fold. It consists of a ring of seven cpn 60 molecules which are assembled into a bucket-shaped complex which stands on an upturned bucket of another identical seven cpn 60 monomers. Each bucket has a hole in the bottom. Cpn 10 also forms heptamers, and is a dome-shaped molecule positioned like a lid on the top of one of the cpn 60 buckets. Misfolded protein enters the mouth of one of the buckets, where it binds to the hydrophobic lining of the cpn 60 cavity, the cpn 10 lid is snapped on, assisted folding takes place, the lid is removed and the protein is released. When the cpn10 lid binds to the cpn 60 bucket the volume of the bucket increases 2-fold. This involves a twisting movement of the bucket, which shears the hydrophobic binding surface of the cpn away from the bound polypeptide, releasing it into the cavity. The inner surface of the bucket is now lined by hydrophilic residues which favour the burial of the hydrophobic surfaces in the substrate protein and so help in the formation of correctly-folded functional proteins. The bucket contains two hinges, one of which is between the upper side (termed the apical domain) and the lower side (the intermediate domain); the second hinge is between the intermediate domain and the bottom of the bucket, called the equatorial domain. During the enlargement of the cavity, there is a 25° downward movement of the intermediate domain onto the equatorial domain, which locks ATP into its site in the bottom of the bucket. At the same time, there is a 65° rotation of the upper hinge and a 90° clockwise twist of the apical domain about its long axis that moves the hydrophobic lining of the bucket to a new position in which part of the surface interacts with mobilised neighbouring apical domains and the remainder binds to one edge (with sequence Ile-Val-Leu) of the cpn10 mobile β-hairpin loop, which hangs down and outwards from the lower surface of each of the seven cpn10s.

The folding process is asymmetric. Initially, the cpn10 lid is on one of the buckets and the unfolded or kinetically trapped folding intermediate polypeptide enters the other bucket (*trans*). Then, in the presence of ATP, the cpn 10 lid is released from the bucket and ends up on the other bucket which has the polypeptide and the ATP in it. This is accompanied by a doubling of the cavity size which is described above. Folding of the substrate polypeptide takes place in the enclosed cavity. Then ATP binds to the

bottom of the other bucket which results in the ejection of the cpn10 lid, and this allows the native, the committed-to-fold, the uncommitted or the kinetically trapped polypeptide to leave. Once the polypeptide is free of the folding machine, it either regains its function or it binds again to the machine for another refolding cycle.

Not all proteins are folded in this way. Those which are larger than 60–70 kDa may not fit into the bucket with the lid on, although one organism, bacteriophage T4, has solved this problem by evolving a lid with a longer mobile loop and a taller dome which can accommodate its large capsid protein (Hunt, 1997). Alternatively, it is possible that local kinetically trapped regions of large proteins might bind in the trans ring and allow correct folding on release (Gordon, 1994). A few proteins, such as actin and tubulin cannot be folded properly by the GroE chaperonin machine and are folded by the TCP1 chaperonins (Gao *et al.*, 1992; Klumpp *et al.*, 1997).

MONOMERIC AND OLIGOMERIC STATES

In addition to heptamers, cpns can form several different multimeric states and also exist, under certain conditions, as monomers. For example, *E. coli* cpn 10 becomes monomeric below a protein concentration of 0.7 μM (Zondlo *et al.*, 1995). The structure of the subunits of cpn10 heptamers is that of an irregular β-barrel. However, isolated GroES monomers having this structure are energetically unfavourable and only marginally stable at room temperature (Boudker *et al.*, 1997). In contrast, *Mycobacterium tuberculosis* cpn10 forms tetramers which can be detected in bacterial lysates (Fossati *et al.*, 1995), and dimers in dilute buffer (Mascagni *et al.*, 1998). Monomeric *M. tuberculosis* cpn 10 is observed below a concentration of either 4.7 μM or 0.47 μM depending on the concentration of ions such as Mg2+ present in solution. In aqueous buffers these monomers give rise to conformational equilibria. However, in solutions of reduced polarity, these monomers show a preference for a structure which contains both α-helices and β-strands (Mascagni *et al.*, 1998). The biological significance of these findings is unknown, but may have relevance to both the secretion of cpns into the extracellular space, and to the non-folding activities of cpns.

Cellular location

Cpns are located within the cytosol of bacteria and within eukaryotic cells they are found in mitochondria and chloroplasts (Gupta, 1996). For many years it was thought that, because of their crucial intracellular folding role, this was their only location. Recently, however, it has become clear that this is not the case. In bacteria, for example, there is evidence that some cpns, such as the cpn 10 of *M. tuberculosis*, are secreted in large amounts into the extracellular space. About 20% of the total protein content in short-term culture filtrates of logarithmically growing *M. tuberculosis* is cpn 10 (Abou-Zeid *et al.*, 1988). A number of bacteria appear to have cpn 60 associated with their external surfaces. Gentle saline extraction removes surface-associated cpn 60 from the oral pathogen *Actinobacillus actinomycetemcomitans* (Kirby *et al.*, 1995) and this protein has been localised on the outer cell wall by immunogold electron microscopy. The cpn 60 (HspB) of *Helicobacter pylori*, which causes stomach ulceration, also appears on the outside of the cell, in the periplasmic space, in the extracellular space and is believed to act as an adhesin (Eschweiler *et al.*,

1993; Cao *et al.*, 1998). Another pathogen, *Haemophilus ducreyi,* which causes the genital ulcer disease, chancroid, expresses chaperonin 60 on its surface and this protein is involved in the binding of the bacterium to epithelial cells (Frisk *et al.*, 1998). Thus, there appears to be a pattern emerging, with cpn 60 having the ability to act as a bacterial adhesin. This has suggested that the chaperonins may be important bacterial virulence factors (Lewthwaite *et al.*, 1998). A similar story may be emerging with eukaryotic cells. Cpn 60 has been detected by immunogold labelling on the surface of viable chinese hamster ovary cells and the human leukaemic CD4-positive T-cell line CEM-55 (Soltys and Gupta, 1997). In addition to surface expression there is evidence for secretion of cpns from mammalian cells. Thus, human cpn10 is thought to be the same molecule as early pregnancy factor, which is found in the serum of pregnant women (Cavanagh, 1996). In addition, human astrocytes secrete a potent anti-apoptotic cpn 60-like molecule (Brenneman and Gozes, 1996).

How do cpns cross the cell membrane? Generally, transported proteins have an N-terminal signal peptide which is recognised by a cytosolic factor that takes it to a receptor on the membrane (Schatz and Dobberstein, 1996). It is likely that translocation across the membrane is in the monomeric rather than oligomeric form, and so dissociation into monomers is required. Cpns do not have a specific α-helical signal peptide, but those that are secreted, like the *M. tuberculosis* cpn 10 have a signal peptide-like structure at the N-terminus which forms an α-helix in organic solvent/buffer mixtures. It is possible that the N-terminus of some cpns help in the translocation of the molecule across the membrane. It is also possible that there may be direct association of C-terminus of cpn 60 with membranes. Torok and colleagues (Torok *et al.*, 1997) have found that cpn 60 can associate with model lipid membranes, and that proteolytic removal of the C-terminus of the protein prevented association with lipid membranes. Interestingly, the cpn 60 increased the lipid order in the liquid crystalline state, which suggests that cpns can assist the folding of both soluble and membrane-associated proteins whilst, at the same time, stabilising lipid membranes.

Importance in medicine

FOLDING ACTIVITY

Damage to tissues occurs when the blood supply is cut off, and is called ischaemia. Mitochondria in particular are damaged under such conditions. Lau and colleagues (Lau *et al.*, 1997) made cpn 60 and cpn10 adenovirus constructs which they over-expressed in rat neonatal cardiomyocytes and in the myogenic H9 c2 cell line. Simultaneous expression of both of these cpns protected cells from simulated ischaemia, but expression of only one of the cpns led to cellular damage. These data suggest that induction of cpn expression in tissues may protect them from damage due to ischaemia which is seen in organs such as the kidney during the transplantation process.

It has also been suggested (Carrell and Lomas, 1997) that a number of diseases might arise from abnormal folding and subsequent aggregation of specific proteins. For example, accumulation of misfolded proteins occurs in spongiform encephalopathy, α-$_1$antitrypsin deficiency-associated lung and liver disease, Alzheimer's disease and amyloidosis. It is not known whether cpns are involved in the pathology of these conditions.

NON-FOLDING ACTIVITY

Bone diseases

The skeleton is the largest organ in the body and is prey to many infections and to idiopathic conditions such as osteoporosis. Bone, although composed largely of extracellular inorganic matrix, is not a dead tissue but is permeated by a rich blood and nerve supply and is being continuously destroyed and rebuilt in a dynamic process called bone remodelling. This ongoing remodelling is due to the action of two cell lineages. Bone matrix removal is 'catalysed' by a multinucleate myeloid cell called an osteoclast, while replacement of this resorbed matrix is the responsibility of the mesenchymal cells called osteoblasts. Bacterial infections cause common bone diseases such as the periodontal diseases (with a prevalence of 10–15% worldwide), osteomyelitis, bacterial arthritis and tuberculosis of bone. The first evidence that cpns could cause bone pathology was the finding that the cpn 60 of the oral pathogen, *Actinobacillus actinomycetemcomitans* could cause bone resorption *in vitro* (Kirby *et al.*, 1995). These studies found that groEL was a potent stimulator of bone resorption but that the cpn 60 molecules of *Mycobacteriun* spp. (hsp 65) were inactive in this respect. The mechanism of action of groEL appears to be its potent ability to stimulate the growth and development of osteoclasts and to activate bone resorption by the mature cells (Reddi *et al*, 1998).

One of the most striking bone pathologies is vertebral tuberculosis or Pott's disease in which the bacterium causes massive destruction of infected vertebrae and gross spinal deformity. Addition of sonicated *M. tuberculosis* to murine bone in culture causes significant loss of bone matrix. Surprisingly, this bone destruction could be completely blocked by a neutralising antibody to *M. tuberculosis* cpn 10 but not cpn 60, confirming previous findings. Recombinant *M. tuberculosis* cpn 10 was a potent inducer of osteolysis which appeared to act as a stimulator of osteoclast proliferation. GroES is also an active osteolytic agent (Nair *et al*, 1998). Using a series of synthetic peptides to define structure/function relationships, the active domains in cpn 10 appear to be the flexible loop and a small loop structure around the conserved tyrosine at position 71. Interestingly, these loop structures make contact with cpn 60 suggesting that if cell activation by cpn 10 is due to receptor binding, then the receptor may have structural similarity to cpn 60 (Meghji *et al*, 1997).

We have recently demonstrated that human recombinant cpn 60 is also able to stimulate bone resorption (unpublished), raising the possibility that idiopathic diseases such as arthritis and osteoporosis could be caused by release of self-chaperonins.

Arthritis

In animals, inflammatory diseases such as arthritis are associated with imbalances in T-cell populations and T-cells which are specific for conserved epitopes of hsp 60 may regulate inflammatory responses (Gaston, 1998). Passive transfer of a T-cell clone which is specific for the 180–188 amino-acid sequence of mycobacterial hsp 60 induces adjuvant arthritis (Holoshitz *et al.*, 1983; Van Eden *et al.*, 1988). In contrast, a synthetic peptide from mycobacterial hsp 60 suppresses adjuvant arthritis and nonmicrobially induced experimental arthritis (Prakken *et al.*, 1998). Similarly, a

synthetic mycobacterial hsp10 peptide delays the onset and severity of adjuvant-induced arthritis (Ragno *et al.*, 1996). These data suggest that hsp10/60 specific T-cells may modulate inflammatory responses in arthritis. Experimental adjuvant arthritis in animals can be induced by the injection of mycobacteria. In humans, a rather similar syndrome occurs after the installation of mycobacteria into the bladder which is part of the treatment for bladder cancer. A rare complication of this treatment is arthritis (Smith *et al.*, 1997; Saporta *et al.*, 1997). The synovial membrane may show cellular infiltrate and cytokine profiles which are similar to those seen in rheumatoid arthritis (Smith *et al.*, 1997). However, the arthritis tends to spontaneously remit, unlike rheumatoid arthritis. In patients with rheumatoid arthritis, hsp 60 is expressed on lymphocytes in peripheral blood and synovial fluid (Sato *et al.*, 1996). These patients also have high levels of antibodies to hsp 60 (Handley *et al.*, 1996) and so, like atherosclerosis (described below), the evidence supports an autoimmune hypothesis. It is possible that antibodies and T-cells arise as a result of an immune response to bacteria such as BCG or *Escherichia coli* (Handley *et al.*, 1996), and that these antibodies recognize cross-reactive epitopes in human hsp 60, so giving rise to microbially-triggered autoimmune damage to the joint. However, complex interactions exist between populations of T-cells in rheumatoid arthritis and suppressive T-cell responses may be induced by human but not by bacterial hsp 60 in the synovial fluid (van Roon *et al.*, 1997). In reactive arthritis due to bacteria such as *Yersinia enterocolitica*, hsp 60 is a powerful antigen which induces CD4+ T populations of cells which are specific to bacterial hsp 60 and others which are potentially autoreactive (Mertz *et al.*, 1998). The link between hsp 60 and arthritis is intriguing but its role in human disease remains to be determined.

Arterial disease

In 1992, Xu and colleagues reported the induction of arteriosclerosis in normocholesterolemic rabbits by immunisation with *M. tuberculosis* cpn 60 (hsp 65) (Xu *et al.*, 1992). Immunisation with hsp 65 combined with a cholesterol-rich diet led to more severe atherosclerosis. The hypothesis which has emerged from these findings proposes that an autoimmune response contributes to the inflammation in atheromatous arteries and that a high blood cholesterol is an important risk factor (Wick *et al.*, 1995). The rationale for this hypothesis is the finding that autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity (Schett *et al.*, 1995; Wick *et al.*, 1995). It seems that these autoantibodies cross-react with mycobacterial hsp 65, human hsp 60 and a 60 kDa protein (probably cpn 60) in heat-shocked endothelial cells. Only heat-stressed endothelial cells were lysed by anti-hsp 60/65 antibody in the presence of complement or peripheral blood mononuclear cells. Is this relevant to human atherosclerosis which kills a high proportion of adults in the Western world? Patients with atherosclerosis have elevated levels of anti-hsp 65 antibodies (Mukherjee *et al.*, 1996; Hoppichler *et al.*, 1996). Macrophages in the atherosclerotic lesions of patients express high levels of hsp 60 and human anti-hsp 60 antibodies induce complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity of stressed peripheral blood derived macrophages and the macrophage-like cell line U937 (Schett *et al.*, 1997). This is not sufficient evidence to prove an association because the raised level of anti-hsp 65 could be a coincidental

finding, which is not related to the underlying pathogenesis of atherosclerosis. However, in rabbits, which have been immunized with mycobacterial hsp 65, there is increased expression of hsp 65 in atherosclerotic lesions and these arterial lesions are infiltrated with T-cells which react with hsp 60/65 (Xu *et al.*, 1993). The serum of the immunized rabbits contains elevated levels of antibody to hsp 65. Interestingly, rabbits which are treated with administration of a 0.2% cholesterol diet also generated anti-hsp 65 antibodies and T-cells which are associated with atheromatous arterial lesions. So, in rabbits at least, there is a model of atheroma which is closely associated with an immune response to hsp 65. Another hypothesis (Gupta *et al.*, 1997; Gurfinkel *et al.*, 1997) suggest that bacterial infection may contribute towards the pathogenesis of atherosclerosis. If this were the case, then raised levels of anti-hsp 60 in patients would not be surprising. In rats, treatment with bacterial cell-wall lipopolysaccharide induces coexpression of hsp 60 and intercellular-adhesion molecule-1 and this leads to increased monocyte and T-cell adhesion to aortic endothelium (Seitz *et al.*, 1996). So, microbial infection might induce hsp 60 expression in arteries, and might also induce anti-hsp 60 cross-reacting antibodies and T-cells which could damage the arterial cell walls. Whether human atherosclerosis is an autoimmune or a microbial disease, or a combination of the two, needs further investigation.

Pro-inflammatory effects

Related to 2 and 3 are reports that the chaperonins can stimulate myeloid cells, lymphocytes and endothelial cells to synthesize and secrete the major pro-inflammatory cytokines – interleukin-1 (IL-1), IL-6, IL-8 and tumour necrosis factor (TNF) (Henderson *et al.*, 1996; Verdegaal *et al.*, 1996). This work has been criticised on the basis that activity may be due to residual lipopolysaccharide (a major cytokine-inducing factor from Gram-negative bacterial cell walls) or to the presence of the many peptides and proteins which co-purify with the chaperonins, particularly cpn 60 (Price *et al.*, 1991). However, in a recent study a method has been developed to prepare groEL to homogeneity and in the absence of contaminating peptides and lipopolysaccharide this protein is still a potent stimulator of cytokine synthesis. In addition, proteolysis of groEL resulting in its breakdown into small peptides did not significantly inhibit its ability to stimulate human monocytes to produce pro-inflammatory cytokines (Tabona *et al.*, 1998). This finding suggests that cpn 60, which is a major component in stressed cells such as those causing inflammation in the host, may be an extremely important virulence factor able to stimulate pro-inflammatory signals even after it has been 'destroyed' by proteolysis

Alzheimer's disease

This common condition is characterised by widespread death of neurones in the brain. A potent molecule, called activity-dependent neurotrophic factor (Gozes and Brenneman, 1996) is secreted by vasoactive intestinal peptide-treated astrocytes. This 14 kDa protein protects neurons from death associated with an Alzheimer's disease model. A short 14-amino acid peptide derived from the protein sequence is active at femtomolar concentrations (Brenneman and Gozes, 1996) and protects toxin-treated

rats from memory loss (Gozes *et al.*, 1997). Interestingly, the protein is homologous to cpn 60. This observation raises the possibility that extracellular cpns have direct potent actions on human cells and that short peptides derived from the cpn sequence also act directly on cells. It is also possible many other examples of direct cpn action on eukaryotic cells exist but are, so far, undetected. Perhaps whilst intracellular cpns protect cells from stress, extracellular cpns transmit protective messages between cells and even from one organ to another, rather like hormones.

The expression of human cpn 60 is upregulated in various human central nervous system diseases and in experimental animals, particularly in astrocytes and abnormal neurones (Martin *et al.*, 1993; Khanna *et al.*, 1996). It has been suggested that autoimmune inflammation in the central nervous system is associated with increased cpn 60 expression and that this might, in some way, modulate the immune response (Gay *et al.*, 1995). One hypothesis (Birnbaum *et al.*, 1996; Birnbaum and Kotilinek, 1997), is that there is a cross-reacting epitope between cpn 60 and the myelin protein 2', 3' cyclic nucleotide 3' phosphodiesterase, and immunisation with a peptide which contains this epitope modifies the course of acute and chronic experimental autoimmune encephalomyelitis. Cpn 10 can also protect animals against experimental autoimmune encephalomyelitis but, in contrast to the cross-reactive cpn 60, has no shared T-cell epitopes with encephalitogenic proteins (Ben-Nun *et al.*, 1995).

Growth regulation

Pregnancy and cancer are, perhaps, the most dramatic natural examples of mammalian cell growth. Chaperonins are implicated as growth regulators in both. Early pregnancy factor which has been identified as cpn 10, is required for the successful establishment of pregnancy and for the proliferation of both normal and neoplastic cells (Cavanagh, 1996). However, it has been questioned whether early pregnany factor is identical with cpn 10 (Clarke, 1997). A so-far unidentified molecule which is called preimplantation factor is detected in the serum of women shortly after fertilisation (Roussev *et al.*, 1996), but this factor seems to be different to cpn 10.

The cpn 10 of *M. tuberculosis* but not of *E. coli*, increases the proliferation of the rapidly growing mouse P19 teratocarcinoma cells. In contrast, the *M. tuberculosis* cpn 10 increases apoptosis in serum-deprived teratocarcinoma cells. This suggests that this cpn 10 has opposite affects on cell growth depending on the condition of the target cell (Galli *et al.*, 1996). In mice which contain the highly malignant reticulum sarcoma (J774), delivery of the mycobacterial cpn 60 (hsp 65) gene DNA in liposomes results in regression of the tumour (Lukacs *et al.*, 1997). The mechanism of action of the cpn 60 is not clear, but the injected animals also produce antibodies against the tumour cells which suggest the tumour becomes more antigenic after gene transfer, and so, perhaps is rejected by the immune system.

Vaccines

Some small molecules such as the Vi Ag of *Salmonella typhi* are poorly immunogenic T-independent antigens. Cpn 60 peptides serve as immunogenic carriers for such antigens and can greatly increase their immunogenicity (Konen-Waisman *et al.*, 1995).

The future

FOLDING

Co-expression of cpns with recombinant proteins may help to increase the yield of correctly folded protein and so make industrial production of proteins more efficient. A rather different line of thought lies behind the idea of pre-stressing transplantation organs, so that they increase their content of cpns and so become more resistant to the rigours of existing without a blood supply.

NON-FOLDING

The potential use of cpns to treat disease is now a growth area. The potency of cpns to directly affect the function of cells such as neurones and bone cells suggests that new pathways of action will be discovered and may prove amenable to therapeutic intervention. The indirect affects of cpns, operating through the immune system, either by suppressing immunity or by enhancing it, will continue to be an active area. New vaccine formulations may well emerge to boost the immune response to specific peptides by combination with cpns.

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