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GRP78 induction by cyclosporin A in human HeLa cells

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Abstract

Immunosuppressive drugs such as cyclosporin A (CsA) and FK506 are known to have pleiotropic effects on cells. Here we demonstrate that treatment of HeLa cells with low concentrations of CsA (but not of FK506) induces the synthesis of a stress protein, GRP78, located inside the endoplasmic reticulum. High concentrations of CsA lead to a general decrease in protein synthesis. When cells are stressed (heat-shocked) during the CsA treatment, the synthesis of heat shock proteins is reinforced. FK506 has no detectable effects at any concentration. The mechanism of induction of GRP78 by CsA remains presently unknown. Whatever the mechanism involved, GRP78 overexpression might be responsible for some of the physiological effects of CsA.

Key words: Cyclosporin A; Glucose regulated protein (GRP78); Peptidyl-prolyl-isomerase; Heat-shock

1. Introduction

Cyclosporin A (CsA) is largely used in medicine as an immunosuppressive drug to avoid the rejection of organ transplants as well as to treat autoimmune diseases. CsA binds to proteins called cyclophilins which belong to a large class of enzymes having peptidyl-prolyl *cis-trans* isomerase (PPI) activities. However, the immuno-suppressive action of CsA does not seem to be directly related to its inhibitory action on PPI activity [1]. The CsA-cyclophilin complex has a specific inhibitory effect on the calmodulin-dependent protein phosphatase calcineurin, which leads to the inhibition of the transcription of lymphokine genes associated with the early phase of T lymphocyte activation [2–4]. Other inhibitory effects of CsA on transcription have been described, distinct from those involved in the control of lymphokine genes [5].

The predominent side effects of CsA treatment occur in the kidney. The mechanisms of nephrotoxicity however remain unclear: CsA reduces the gluconeogenic capacity of net proximal tubules via a specific decrease in the transcription of the PEPCK gene (phosphoenol pyruvate carboxykinase) in this tissue [6]; on the other hand, CsA increases, both in vivo and ex vivo, the expression of procollagen genes in kidneys [7,8].

Our project was to use CsA (and another immunosuppressive drug, FK 506) to approach the physiological significance of the PPI activities. PPIs have been characterized in prokaryotes as well as in eukaryotes. They are abundant enzymes, present in all cell compartments. They belong to two different structural families which are the respective targets of CsA on the one hand, FK506 and rapamycin on the other. In vitro, their addition increases the rate of folding for some proteins such as ribonuclease T1 or immunoglobulin light chains [9,10]. The in vivo physiological significance of the PPIs remains however elusive [11]. Deletion of the different PPI genes has no or limited consequence in yeast [12,13]. However PPIs might be required in vivo for the proper folding of some proteins [14–16]. Recently PPIs of the cyclophilin subfamily have been shown to interact with the gag protein of HIV, and this interaction is suggested to be necessary for viral infectivity [17,18].

If PPIs are essential for the correct folding of a sufficient amount of proteins, their inhibition by immunosuppressive drugs ought to lead to the accumulation of denatured proteins in different cell compartments. These denatured proteins would switch on or increase the level of the stress response and the subsequent synthesis of HSPs (or GRPs if the denatured proteins accumulate in the endoplasmic reticulum [19–22]. Therefore we decided to look at the effects of FK506 or CsA on the synthesis of stress proteins, in the absence or following a heat treatment. It was recently demonstrated that the level of mRNAs corresponding to the major cytoplasmic cyclophilin genes in yeast is increased by heat treatment [23]. Moreover the FKB2 gene of *Saccharomyces cerevisiae*, encoding FKBP-13, is induced by heat-shock and by the accumulation of unfolded proteins in the endoplasmic reticulum [24]. Both observations suggest that PPIs may be involved in the repair of heat-shock damage and reinforce the rational basis of our approach.

2. Materials and methods

2.1. Cell culture and heat shock

MRL2 is a clone of HeLa cells. CHO cells were kindly provided by the laboratory of Gérard Buttin, Institut Pasteur. All cells were propagated in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supple-

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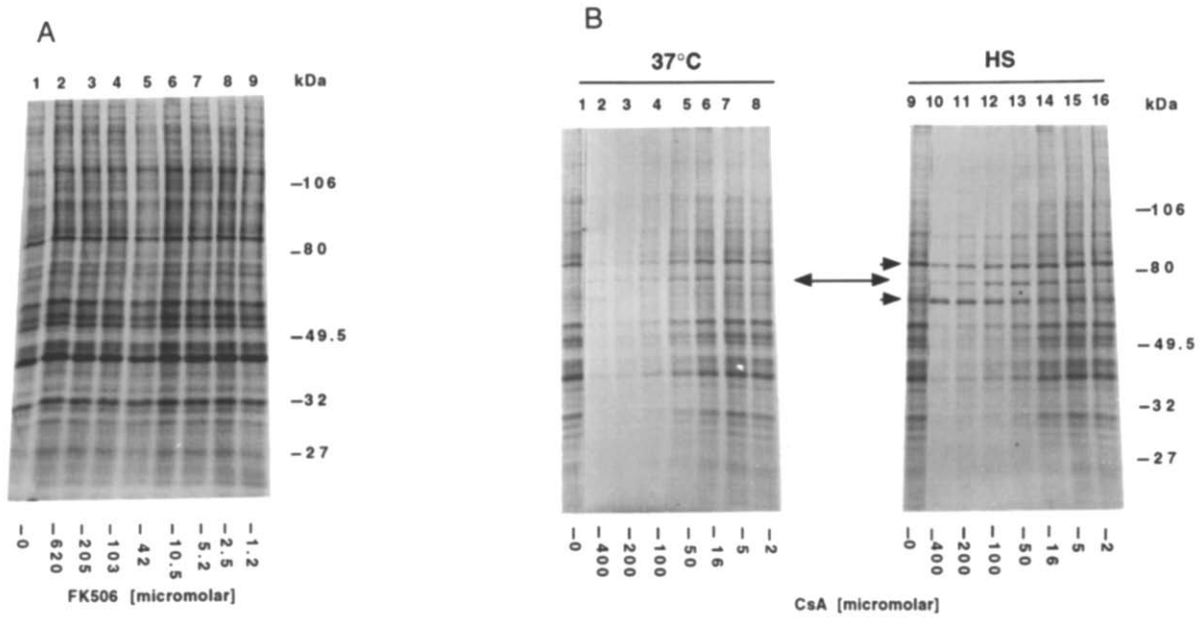

Fig. 1. Modification of protein synthesis induced in heat-shocked and non-heat-shocked HeLa cells by FK506 and CsA. (A) Incubation with variable concentrations of FK506. MRL2 cells were incubated in absence of FK506 (lane 1) or in presence of different concentrations of FK506 (lanes 2–9) for 5 h. [^{35}S]Methionine was added for the last hour. (B) Incubation with variable concentrations of CsA. In lanes 1–8, MRL2 cells were incubated for 4 h with (or without, lane 1) CsA, then supplemented with [^{35}S]methionine for 1 h. In lanes 9–16, cells were incubated for 30 min with (or without, lane 9) CsA, then submitted to a 30 min heat-shock at 44°C, followed by incubation at 37°C for 3 h. [^{35}S]Methionine was added for 1 h (in all the experiments, the total duration of the incubation with CsA was 5 h). Cells were collected and analyzed as previously described. Arrows point to the 75 kDa protein induced by CsA. Arrowheads point to the major 90 kDa and 70 kDa HSPs. The concentrations of the different drugs are indicated below the different lanes (in micromolar).

mented with 10% fetal calf serum. Cells were plated at a low density in 24-well plates and incubated with the different drugs during the exponential growth phase. CsA or FK506 were prediluted in cell culture medium and incubated with cells for 5 h (unless otherwise stated). During the last hour of incubation, [^{35}S]methionine was added (300 μCi/ml; 120 μCi/well). Cells were rapidly washed with PBS, resuspended in 50 μl of Laemmli sample buffer and the samples heated for 10 min at 90°C.

The heat shock treatment was performed by immersing the culture dishes in a waterbath at 44°C during 30 min. The drugs (CsA or FK506) were added to the cells 30 min before the beginning of the heat treatment. After it, the cells were brought back to 37°C for 4 h. During the last hour of incubation at 37°C, [^{35}S]methionine was added as previously described.

The induction of GRPs was obtained by adding the ionophore A23187 at 7 μM final concentration for 5 h.

2.2. 1- and 2-dimensional gel electrophoresis

The different samples were analyzed by SDS–10% polyacrylamide gel electrophoresis [25]. 20 μl of each sample, corresponding to the same amount of cells, is deposited on each lane. The gels are fixed, dried and submitted to autoradiography. The molecular masses of the different proteins were checked by using the prestained SDS-PAGE Standards from Bio-Rad (low range).

Isoelectrofocusing was performed with 8 cm polyacrylamide gels containing 1% 3.5–10 and 1% 5–8 ampholines (Pharmacia). 350 V were applied to the gel tubes for 16 h, and 800 V for 10 min at the end of the experiment. 1D-gels were deposited at the top of SDS–10% polyacrylamide gel, run and treated as previously described.

3. Results

3.1. Addition of CSA leads to the induction of GRP78

MRL2 cells were incubated with CsA and FK506 for

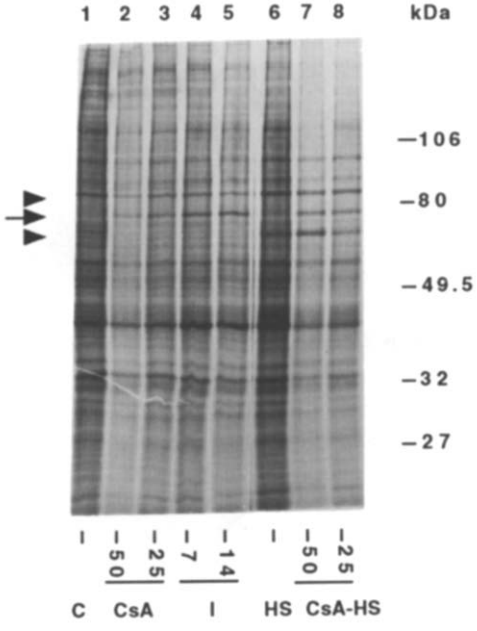

Fig. 2. The major protein induced by CsA comigrates with the protein induced by the addition of ionophore A23187. MRL2 cells were incubated for 4 h at 37°C (C), with CsA (lanes 2 and 3), with calcium ionophore A23187 (I) (lanes 4 and 5), incubated 30 min at 37°C, 30 min at 44°C and then 3 h at 37°C (lane 6), as lane 6 but in presence of CsA (lanes 7 and 8). [^{35}S]Methionine was added for 1 h and cells were treated as in Fig. 1. Arrows point to the 75 kDa protein induced by CsA. Arrowheads point to the major 90 kDa and 70 kDa HSPs. The concentrations of the different drugs are indicated below the lanes.

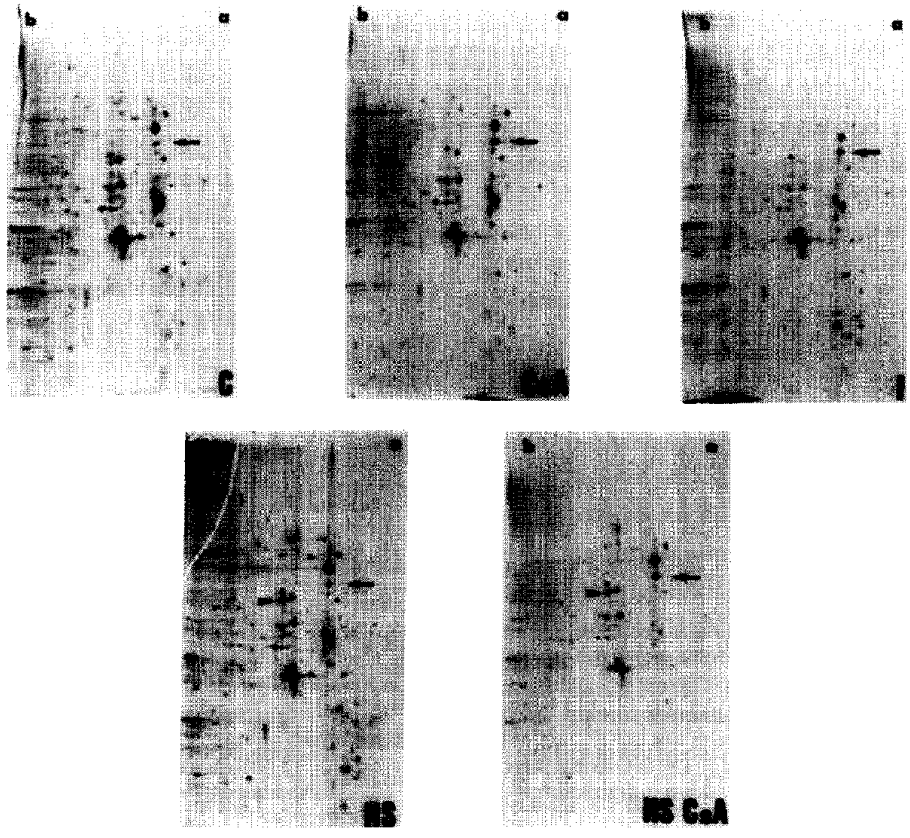

Fig. 3. Identification of the protein induced by CsA as GRP78 by 2D gel electrophoresis. MRL2 cells were incubated for 4 h at 37°C (C); for 4 h at 37°C with 25 μM CsA (CsA); for 4 h at 37°C with 7 μM ionophore A23187 (I); at 37°C for 30 min, brought to 44°C for 30 min and then to 37°C for 3 h (HS); with 25 μM CsA at 37°C for 30 min, brought to 44°C for 30 min and back to 37°C for 3 h (HS CsA). [^{35}S]Methionine was then added for 1 h; cells were lysed and proteins analyzed by 2D gel electrophoresis, followed by autoradiography. Arrows point to GRP78. Arrowheads point to the 70 kDa major HSP. a and b correspond, respectively, to the acidic and basic pI. In all the experiments, the total duration of the incubation with CsA or the ionophore was 5 h.

4 h. [^{35}S]Methionine was then added for 1 h. Cells were rapidly lysed in SDS-containing buffer and analyzed by 1-dimensional gel electrophoresis. No modifications in protein synthesis can be observed with FK506, whatever the concentration used (between 1 and 600 μM) (Fig. 1A). On the contrary, CsA deeply alters protein synthesis (Fig. 1B). At high concentration (higher than 50 μM), CSA leads to a general decrease in protein synthesis. Even at low doses of CsA (2 μM), it is possible to observe an increase in the synthesis of a protein of about 75 kDa. This protein can be induced by concentrations of CsA lower than 1 μM (data not shown).

The position of this protein in 1D gel electrophoresis was reminiscent of the position of the glucose regulated protein GRP78. Since GRP78 and other GRPs can be specifically induced by addition of calcium ionophore such as A23187, we compared the migration of the protein induced by CsA with the migration of the protein induced by A23187 (Fig. 2). The two proteins comigrate exactly in 1D gel electrophoresis, suggesting that the protein induced by CsA was identical to GRP78.

The identification of the protein induced by CsA treatment with GRP78 was confirmed by 2D gel electrophoresis (Fig. 3). The position of GRP78 in 2D gel electrophoresis is very characteristic. It migrates at the same pI as tubulins just below the doublet of HSP90. Incubation with ionophore A23187 leads to a general decrease in protein synthesis, but also to a clear induction of GRP78 (Fig. 3, I). Addition of CsA leads also to an increase in GRP78 synthesis (Fig. 3, CsA). Heat shock alone does not induce GRP78 synthesis in a significant way (Fig. 3, HS).

The maximal level of induction of GRP78 ny CsA is obtained only 3 h after the beginning of the incubation. Longer exposures to CsA (15 h) do not lead to an increase in the induction of GRP78 (data not shown).

The induction of GRP78 following treatment with CsA is also observed in Chinese hamster ovary cells. However the high constitutive level of GRP78 in the latter limits the factor of induction observed.

We were intrigued by the differential actions of CsA and FK506. We checked the possibility that FK506 action would be revealed in association with CsA. Such was not the case: the response to CsA was not modified by the addition of two different doses (4 and 40 μM) of FK506 (Fig. 4).

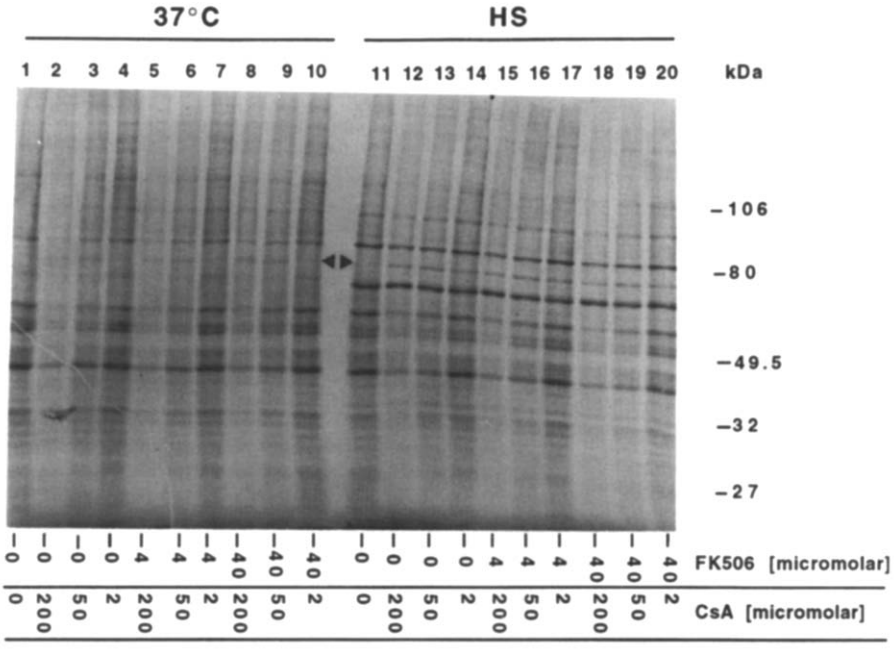

Fig. 4. Addition of FK506 does not alter the modifications of protein synthesis induced by CsA. In absence of heat-shock (lanes 1–10): cells were incubated for 4 h at 37°C (lane 1), with CsA in the absence (lanes 2–4) or presence (lanes 5–10) of FK506. After a heat-shock (lanes 11–20): cells were incubated for 30 min at 37°C, 30 min at 44°C and brought back to 37°C for 3 h in the absence of CsA (lane 11) with CsA in the absence (lanes 12–14) or presence (lanes 15–20) of FK506. With and without heat shock: [^{35}S]methionine was added in all dishes for 1 h. Cells were treated as described in Fig. 1. The concentrations of the different drugs are indicated below the lanes. Arrowheads point to GRP78.

3.2. *CsA does not induce the heat shock response, but strengthens the synthesis of HSPs observed after a heat treatment*

The previous experiments showed that GRP78 was induced by preincubation with CsA. On the contrary, the level of the major HSPs (HSC73, HSP72 and HSP90) was unaltered (Fig. 1B).

However when high concentrations of CsA were added before the heat treatment (44°C for 30 min), CsA had a clear effect on the synthesis of HSPs (Fig. 1B). Since the general protein synthesis is deeply reduced by the synergic action of heat treatment and high concentrations of CsA, a slight increase in the absolute level of HSP synthesis leads to a dramatic increase in the relative level of 70 kDa and 90 kDa HSP synthesis (Fig. 1B, and Fig. 2, lanes 6–8). Identification of HSPs was confirmed by 2D gel electrophoresis (Fig. 3, HS and HS CsA).

Moreover the effects observed for CsA on the synthesis of GRP78 in absence of heat treatment were also observed and even increased after a heat treatment: low concentrations of CsA lead to the induction of GRP78 whereas, as previously mentioned, high concentrations decrease protein synthesis. As already observed in the absence of heat treatment, FK506 does not modify the pattern of protein synthesis (data not shown). Its concomitant addition with CsA does not alter the effects observed with CsA alone (Fig. 4).

Similar observations were done on hamster CHO cells: high concentrations of CsA increase the relative level of HSP synthesis after a heat shock.

4. Discussion

CsA is not able by itself to induce the synthesis of HSPs in agreement with what was previously observed [26]. Maybe the amount of cytoplasmic proteins requiring the catalysis of X-Pro bond isomerization for their correct folding is too limited to induce HSP synthesis. However, after a heat treatment, addition of relatively high doses of CsA leads to an increased synthesis of HSPs and to a decreased synthesis of the other proteins. Therefore the action of CsA results in a reinforcement of the heat shock response. This might be due to increase in the triggering signal of the heat shock response, i.e. the accumulation of unfolded proteins inside cells. However this stimulating effect of CsA is observed at high concentrations, reducing the physiological significance of the latter observation.

What are the mechanisms leading to the induction of GRP78 at low concentrations of CsA? The most attractive hypothesis is that inhibition of PPIs is responsible for the misfolding of proteins inside the endoplasmic reticulum. This accumulation of misfolded proteins would lead to the induction of GRPs. Experiments are now in progress to demonstrate that the increase in GRP78 synthesis after CsA treatment results from the transcriptional activation of the corresponding gene.

The striking difference between the significant effects observed with CsA and the lack of effects of FK506 might be explained by the different enzymatic properties of cyclophilins and FK506 binding proteins [27]. Cyclo-

philins are non-specific PPIs, able to isomerize all X-Pro bonds. On the opposite, FK binding proteins catalyze specifically the isomerisation of a limited set of X-Pro bonds. Therefore inhibition of FKBP can be by-passed by the action of cyclophilins whereas inhibition of cyclophilins cannot be rescued by the specific activity of FKBP.

The previous observations show that low concentrations of CsA may lead to limited, but significant alterations in protein synthesis. Therefore an increase in GRP78 level might be involved in the different physiological effects of CsA.

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