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Indirect participation of Hsp90 in the regulation of the cyclin E turnover

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ABSTRACT

Cyclin E is the Cdk2-regulatory subunit required for the initiation of DNA replication at the G1/S transition. It accumulates in late G1 phase and gets rapidly degraded by the ubiquitin/proteasome pathway during S phase. The degradation of cyclin E is a consequence of its phosphorylation and subsequent isomerization by the peptidyl-prolyl isomerase Pin1.

We show that in the colon cancer cells HT-29 the inhibition of the chaperone function of Hsp90 by geldanamycin (GA) enhances the ubiquitinylation of cyclin E and triggers active degradation via the proteasome pathway. As Hsp90 forms multiprotein complexes with and regulates the function and cell contents of numerous signaling proteins, this observation suggests a direct interaction between Hsp90 and cyclin E. However, experiments using cell lysate fractionation did not reveal the presence of complexes containing both Hsp90 and cyclin E. Coupled transcription/translation experiments also failed to detect the formation of complexes between newly synthesized cyclin E and Hsp90. We conclude that Hsp90 can regulate the degradation of cellular proteins without binding to them, by an indirect mechanism. This conclusion postulates a new category of proteins that are affected by the inactivation of Hsp90. Our observations do not support the possible involvement of a PPIase in this indirect mechanism. Besides, we did not observe active geldanamycin-dependent degradation of cyclin E in the prostate cancer-derived cell line DU-145, indicating that the Hsp90-dependent stabilization of cyclin E requires specific regulatory mechanism which may be lost in certain types of cancer cells.

1. Introduction

Progression through the cell cycle in normal eukaryotic cells is tightly controlled by the expression of cyclins and activation of cyclin-dependent kinases. Cyclin E is expressed in the late G1 phase and promotes events associated with the G1/S transition by activating Cdk2, its associated kinase [1–5]. During S phase cyclin E undergoes a phosphorylation-dependent proteolysis [6] carried out by the ubiquitin-proteasome pathway [7,8] and Cdk2 activity is further dependent on binding to cyclin A.

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Abbreviations: Hsp90, heat shock protein 90; GA, geldanamycin; MG132, proteasome inhibitor (*N*-benzoyloxy-carbonyl (Z)-Leu-Leu-leucinal); CHX, cycloheximide; Cdk, cyclin-dependent kinase; PPIase, peptidylprolyl isomerase; PMSF, phenyl methyl sulfonyl fluoride; PBS, phosphate-buffered saline; TBS, tris-buffered saline; DMEM, Dulbecco's modified Eagle's medium; MOPS, 3-morpholinopropane-1-sulfonic acid; IPTG, isopropyl-beta-D-thiogalactopyranoside; IMAC, immobilized metal affinity chromatography.

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Cells respond to stress by the expression of ''heat shock proteins'' (Hsp), a family of molecular chaperones (reviewed in [9]). Hsp90 is a key member of this family, ubiquitously expressed also in the absence of stress. Inhibition of Hsp90 leads to stress response including the induction of, for instance, Hsp27 and Hsp70 [10]. Hsp90 acts as a chaperone toward numerous proteins, most of which are involved in signal transduction [11,12]. By forming multiprotein complexes with various co-chaperones, Hsp90 controls the balance between folding/maturation and proteasomal degradation of its client proteins [13]. This role is particularly important for the viability of transformed cells: indeed, the expression of Hsp90 is enhanced in tumor cells and favors their survival by preventing the aggregation of mutated proteins that would be otherwise toxic [9,14,15].

Hsp90 is the unique known cellular target of geldanamycin (GA), a benzoquinone ansamycin [16], a drug which acts by binding to the ATP-binding site at the amino-terminal domain of Hsp90 [17] with a higher affinity than ATP or ADP [18–20]. GA inactivates the chaperone function of Hsp90 and favors client protein degradation via the ubiquitin/proteasome pathway [20–22]. It has been reported that in tumor cells Hsp90 has a much higher affinity for GA than in normal cells [23]. GA is a promising anticancer agent currently tested in clinical trials [24].

We previously showed [25] that GA induced a cell cycle arrest in the G1 and/or G2/M phases depending on the cell type and cell culture conditions. This antiproliferative effect of GA was accompanied by a rapid inhibition of the cyclin E gene expression and cyclin E/Cdk2 kinase activity, together with the inhibition of the transcriptional activity of the cyclin E gene promoter. In this work we show that, in addition to its negative effect on the activity of the cyclin E gene promoter, GA induces a rapid and active degradation of the cyclin E protein through enhanced ubiquitinylation.

2. Materials and methods

2.1. Reagents, antibodies and constructs

Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for anti-cyclin E (C-19; sc-198), mouse anti-cyclin E monoclonal antibody (HE-12; sc-247), anti-Cdk2 (M2; sc-163), anti-Hsp90 (H-114; sc 7947), anti-cyclin A (C-19; sc-596) anti-actin-HRP (C11; sc-1615), anti-ubiquitin (FL-76; sc-9133), anti-β-catenin (E-5; sc-7963) and from Clontech (Palo Alto, CA, USA) for anti-cyclin D1. Anti-Flag M2 monoclonal antibody (F 3165) was from Sigma–Aldrich (St. Louis, MO, USA). Secondary reagents (HRP conjugates) and non-immune rabbit immunoglobulin (IgG) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ni^{2+}-NTA agarose beads were from Qiagen (Valencia, CA, USA). Redivue L-[^{35}S]Methionine (1000 Ci/mmol) and protein G-Sepharose beads were purchased from Amersham Biosciences (GE Healthcare, Europe, Orsay, France). The TNT-Quick Coupled Transcription/Translation system was from Promega (Madison, WI, USA). NuPAGE® Novex Bis–Tris Gels (Novex, San Diego, CA, USA) and MOPS buffer were purchased from Invitrogen (Invitrogen SARL, Cergy Pontoise, France). The full-length cyclin EL-FLAG-tagged construct cloned in pcDNA3.1 vector was a generous gift of Pr. K. Keyomarsi (University of Texas, Houston, TX 77030). Hexahistidine Hsp90 protein [$(His)_6$-Hsp90] was produced from *Escherichia coli* BL21-Gold strain transformed with the pET-28 vector (Novagen, Merck Chemicals, Nottingham, UK) bearing the entire cDNA coding sequence of Hsp90 [27] cloned in frame downstream of the hexahistidine sequence. Geldanamycin (GA), MG132, cycloheximide (CHX) were purchased from Calbiochem (La Jolla, CA, USA), aphidicolin, IPTG and kanamycin from Sigma Chemical Co. (St. Louis, MO, USA). Protein concentrations in cell extracts were evaluated using the BCA Kit (Bio-Rad, Hercules, CA, USA). All other laboratory chemicals were of reagent grade, purchased from standard suppliers.

2.2. Cell culture

The human cancer-derived cell lines HT29 (colon cancer) and DU145 (prostate cancer) were cultured in DMEM medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS; BioWest, Nuaillé, France) and antibiotics (penicillin 50 U/ml; streptomycin 50 μg/ml) at 37 °C, in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were treated by GA (500 nM) either during exponential growth or after synchronization at the G1/S transition by aphidicolin (2 μg/ml, 17 h). When indicated, the cells were exposed to CHX (10 μg/ml) to block protein synthesis, or to MG132 (10 μM) to inhibit protein degradation by the proteasome pathway.

2.3. Detection of cellular proteins

Cells were washed twice with PBS and scraped in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 0.2 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT). After 15 min on ice, insoluble debris were removed by centrifugation (12 000 × *g* 5 min at 4 °C). For Western blotting analysis, 100 μg protein portions of cell lysates were boiled in Laemmli sample buffer (25 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue), for 3 min. The proteins were separated by SDS-PAGE and electro-transferred to nitrocellulose membranes. After blocking with PBS-Tween 0.1% containing 5% non-fat dried milk, the membranes were probed with specific antibodies, washed with PBS-Tween 0.1% and revealed with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibodies. Specific protein bands were visualized with the ECL detection reagent (Amersham, GE Healthcare Europe, Orsay, France).

For immunoprecipitation, portions of cell lysates (300 μg protein) were incubated for 2 h on ice with the specific primary antibody with sporadic manual shaking and then rotated overnight with protein G-Sepharose beads at 4 °C. After centrifugation, the pellets were washed three times with lysis buffer, then boiled in Laemmli sample buffer for 3 min and resolved by SDS-PAGE and analyzed as described above.

2.4. Gel filtration chromatography

HT-29 cells were synchronized by aphidicolin and treated with GA as described in the text. The cells were scraped in lysis

buffer supplemented or not with 20 mM Na_2MoO_4. Samples (50–100 μl) containing 400 μg of protein were loaded on a Superdex-200 column (Superdex 200 PC 3.2/30; GE Healthcare Europe, Orsay, France) equilibrated with buffer (TBS, 2 mM EDTA, 20 mM Na_2MoO_4). The elution was carried out at 10 °C, with elution buffer (TBS, 50 mM NaCl, 2 mM EDTA, with or without 20 mM Na_2MoO_4) flow rate 40 μl/min; fractions of 50 μl were collected and 20 μl aliquots were analyzed by SDS-PAGE and Western blotting as described above.

2.5. *Expression and purification of recombinant $(His)_6$-Hsp90*

Hexahistidine-tagged Hsp90 [$(His)_6$-Hsp90] protein was expressed in *E. coli* BL21-Gold cells grown in LB medium with kanamycin (30 μg/ml), at 37 °C and induced with IPTG (0.5 mM) overnight at 20–22 °C. Cell pellets (12 000 × *g* 10 min, at 4 °C) were resuspended in lysis buffer (100 mM Na-phosphate pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.2 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT). The bacterial lysate, treated with a lysozyme solution (1 mg/ml), was sonicated in 500 mM NaCl, 2% Tween 20 and 10 mM imidazole, and cleared by centrifugation (10 000 × *g* 30 min, 4 °C). The recombinant $(His)_6$-Hsp90 protein was purified by IMAC [28]. Briefly, the supernatant was incubated at 4 °C for 90 min with Ni^{2+}-NTA agarose beads (50% slurry), poured into a column and washed sequentially with elution buffer (25 mM Hepes pH 7.5, 100 mM NaCl) containing increasing concentrations of imidazole. The recombinant protein eluted from the column at 125 mM imidazole, as detected by variations of absorbance at 280 nm. The fractions containing the $(His)_6$-Hsp90 protein were pooled and the protein concentration was determined using the Bio-Rad protein assay and the purity was evaluated by SDS-PAGE and Western blotting.

2.6. *In vitro transcription/translation of cyclin E in the presence of $(His)_6$-Hsp90*

The pcDNA3.1 cyclin E-FLAG construct was transcribed/translated using the TNT Quick coupled reticulocyte lysate system from Promega (Madison, WI, USA), according to the manufacturer's instructions. In brief, the reaction mixtures containing: 1 μg of pCDNA3.1 plasmid including the cyclin E-FLAG insert, 40 μl of TNT Quick Master Mix, 42 μCi of [^{35}S]Methionine and, as indicated, 57 nM $(His)_6$-Hsp90 (or not) and/or 500 nM GA, were incubated at 30 °C for 90 min. The reaction mixtures containing the exogenous $(His)_6$-Hsp90 protein were diluted with (25 mM Hepes pH 7.5, 500 mM NaCl, 10 mM imidazole, 2%Tween 20). The recombinant $(His)_6$-Hsp90 protein was purified by IMAC as described above and eluted with 125 mM imidazole in elution buffer. The reaction mixtures containing no exogenous Hsp90 protein were diluted with (50 mM Tris–HCl pH 7.5, 150 mM NaCl), incubated with anti-Hsp90 or non-immune rabbit IgG for 1 h at room temperature and then overnight with protein G-Sepharose beads at 4 °C. The immune complexes were released by incubating the beads with 10 mM DTT for 30 min at 37 °C and diluted 1/10 with elution buffer [29]. The supernatants containing the immunoprecipitated Hsp90 proteins collected by IMAC or by immunoprecipitation were subjected to a second round of immunoprecipitation with anti-Flag antibody. The protein/antibody mixtures were incubated with protein G-Sepharose beads overnight at 4 °C. The beads were pelleted, washed three times with buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl) or (25 mM Hepes pH 7.5, 100 mM NaCl, 10 mM imidazole) and then boiled in Laemmli sample buffer. Samples were resolved by SDS-PAGE using the NuPAGE® Pre-Cast Gel system developed in MOPS buffer for 50 min. Once dried the gels were exposed at −80 °C on a Kodak X-OMAT film to reveal [^{35}S]Methionine labeled proteins.

3. Results

3.1. *GA induces active degradation of cyclin E*

In earlier experiments [25] we showed that 500 nM GA induced cell cycle arrest in exponentially growing HT29 and DU145 cells. We examined the levels of cyclin E in these cell lines as a function of time of treatment with GA. In the HT29 cells the content of the cyclin E protein was decreased as early as 3 h of incubation with GA, and continued to fall further until at least 9 h. The effect of GA on cyclin E level was abolished when protein synthesis was blocked by CHX (Fig. 1). This result indicates that GA induces an active degradation of the cyclin E protein by a process that requires one or more short-lived protein factors. In contrast, only a slight decrease of cyclin E was seen in DU145 cells after 9 h treatment with GA. In these cells, GA apparently does not cause active degradation of cyclin E.

To examine the possibility that cyclin E is degraded via the proteasome pathway in GA treated HT29 cells as it is the case for other Hsp90 targets [30,31], exponentially growing HT29 cells were treated with 500 nM GA in the presence or absence of 10 μM MG132, a specific proteasome inhibitor. The cell contents of cyclin E and cyclin A, both partners of Cdk2, were analyzed by Western blot (Fig. 2). GA induced a strong decrease of cyclin E that was prevented by MG132. GA had no effect on cyclin A level whereas CHX induced a strong reduction of cyclin A content. The presence of MG132 had no effect on the cyclin A level but stabilized cyclin E during the incubation with GA. Thus, the degradation of the cyclin E protein induced by GA is selective and dependent on the proteasome pathway.

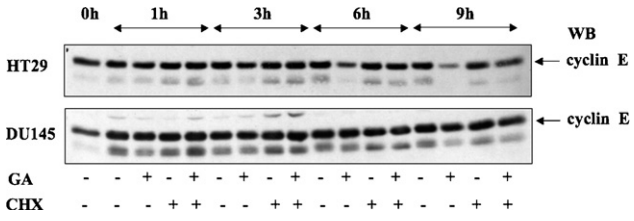

Fig. 1 – GA induces active degradation of cyclin E in HT29 cells. Exponentially growing HT29 and DU145 cells were treated with GA (500 nM) and/or CHX (10 μg/ml) as indicated. Cells were harvested at the indicated time points and the lysates analyzed by SDS-PAGE and Western blotting for cyclin E.

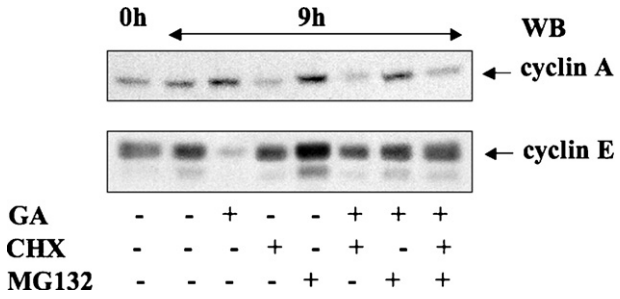

Fig. 2 – Involvement of protein synthesis and of proteasome in the effect of GA. Exponentially growing HT29 cells were treated with GA (500 nM) in the presence of inhibitors of protein synthesis (CHX, 10 μg/ml) or of proteasome function (MG132, 10 μM) for 9 h. Cells were harvested and the lysates analyzed by SDS-PAGE and Western blotting. Cyclin E and cyclin A were revealed by probing the membrane with specific antibodies.

3.2. *GA favors ubiquitinylation of cyclin E in HT29 cells synchronized at the G1/S interface*

During the cell division cycle cyclin E peaks at the G1/S interface. In order to eliminate the possible variations in the content of cyclin E due to the mechanisms linked to the cell cycle progression, we synchronized the HT29 cells with aphidicolin. Cells were then treated with GA and/or MG132 during variable time periods as indicated in Fig. 3 while aphidicolin was maintained in the culture medium to prevent the cell cycle-dependent degradation of the cyclin E, which normally takes place in the S phase. Kinetics of cyclin E degradation in synchronized cells as shown by Western blot analysis of the lysates is in accordance with the results obtained with exponentially growing cells (Fig. 3A).

To verify the implication of the proteasome pathway in the GA-induced degradation of cyclin E we tested the ubiquitinylation of cyclin E. Cyclin E immunoprecipitates obtained from total lysates were analyzed by Western blotting with anti-ubiquitin antibody (Fig. 3B). Treatment of the cells with MG132 induced an accumulation of ubiquitinylated forms of the cyclin E protein (lanes 4, 8, 12) that was further increased in the presence of GA, especially at shorter times (3 and 6 h, lanes 5 and 9).

3.3. *Gel filtration does not show Hsp90-cyclin E association*

GA has been shown to disrupt complexes formed between Hsp90 and its client proteins [32,33], and the degradation of cyclin E during exposure of cells to GA suggests that it may be a client of Hsp90. Thus, we set out to verify whether cyclin E formed a complex with Hsp90. Direct immunoprecipitations with specific antibodies directed against cyclin E or Hsp90 did not give consistent results (data not shown), due to the extremely high cellular concentration of Hsp90 and to its tendency to associate non-specifically with the pellet of antibody-carrying agarose. Next, we subjected lysates prepared from cells treated or not with GA to gel filtration on a Superdex 200 column. Eluted fractions were analyzed by

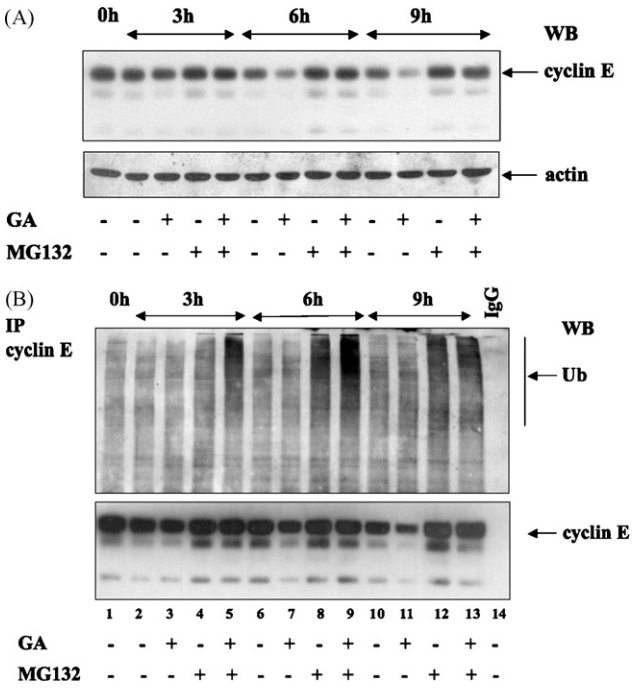

Fig. 3 – Effect of GA on cyclin E degradation in cells synchronized at the G1/S boundary. Exponentially growing HT29 cells were synchronized at the G1/S transition by treatment with aphidicolin (2 μg/ml) for 17 h (t = 0). Aphidicolin was maintained throughout the experiments. GA (500 nM) and MG132 (10 μM) were added to the culture medium at t = 0. Cells were harvested after various periods of time as indicated. (A) Lysates were subjected to SDS-PAGE and Western blotting analysis. Blots were probed with anti-cyclin E and anti-actin antibodies. (B) Lysates were subjected to immunoprecipitation with anti-cyclin E antibody (C-19). Immunoprecipitates were analyzed by SDS-PAGE and Western blotting. Blots were probed with anti-cyclin E (HE-12) and anti-ubiquitin (FL76) antibodies.

Western blotting. The bulk of cyclin E eluted independently of Hsp90 (Fig. 4). There is a narrow zone where both Hsp90 and cyclin E can be detected, so that this approach does not entirely eliminate the possibility that a small portion of cyclin E may form a complex with Hsp90. However, the profiles were identical for lysates of cells treated or not with GA, and we conclude that the effect of GA cannot be ascribed to a dissociation of Hsp90/cyclin E complexes.

Sodium molybdate is known to stabilize the complexes of Hsp90 with certain of its client proteins [34]. The presence of 20 mM Na_2MoO_4 during cell lysis and fractionation had no effect on the elution profiles of either Hsp90 or cyclin E (data not shown).

3.4. *Newly synthesized cyclin E does not form GA-sensitive complexes with Hsp90*

To directly address the question whether cyclin E could be an Hsp90 client protein, we looked for the existence of complexes formed between newly synthesized cyclin E and Hsp90 as it

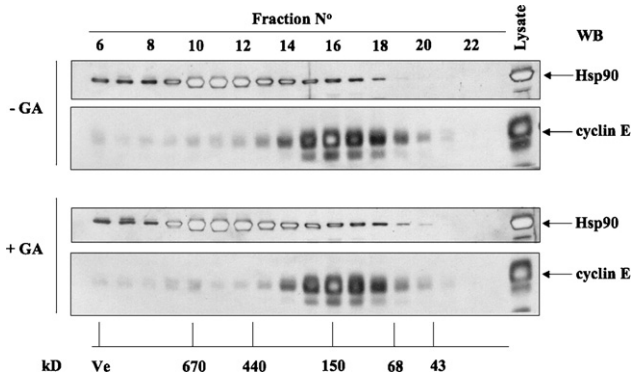

Fig. 4 – Cyclin E and Hsp90 do not co-elute in size fractionation chromatography. HT29 cells were synchronized by aphidicolin at the G1/S transition and treated for 3 h with GA (500 nM) or not, as indicated. Cells were harvested in lysis buffer and the lysates were subjected to size fractionation on Superdex 200. Fractions were analyzed by Western blotting for the presence of cyclin E and Hsp90. The position of protein size markers is shown.

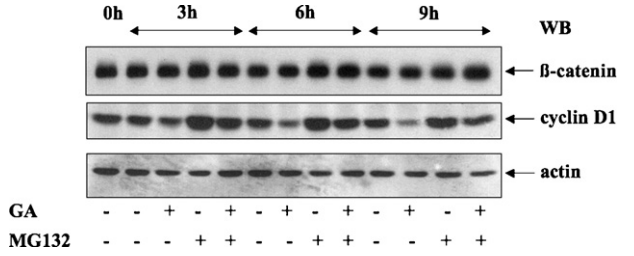

Fig. 6 – Effect of GA on cyclin D1 and β-catenin contents. HT29 cells were treated as in Fig. 3. Cells were harvested as indicated and lysates were analyzed by Western blotting for cyclin D1 and β-catenin contents. GA induced a decrease in cyclin D1 and no change in β-catenin.

was shown for other proteins [35]. In vitro transcription/translation of the Flag-tagged-cyclin E construct was realized in the presence of [^{35}S]Methionine and in the presence or absence of exogenous Hsp90 [$(His)_6$-Hsp90]. The reaction mixtures were subjected to capture of Hsp90 and reimmunoprecipitation with anti-Flag antibody as described in Section 2. Immunoprecipitates were subjected to SDS-PAGE and membranes were analyzed by autoradiography. A stronger signal of [^{35}S]cyclin E was observed as associated with the endogenous Hsp90 than with the exogenous $(His)_6$-Hsp90. In both cases, GA failed to prevent this association, indicating that no specific complexes between cyclin E and Hsp90 were formed (Fig. 5).

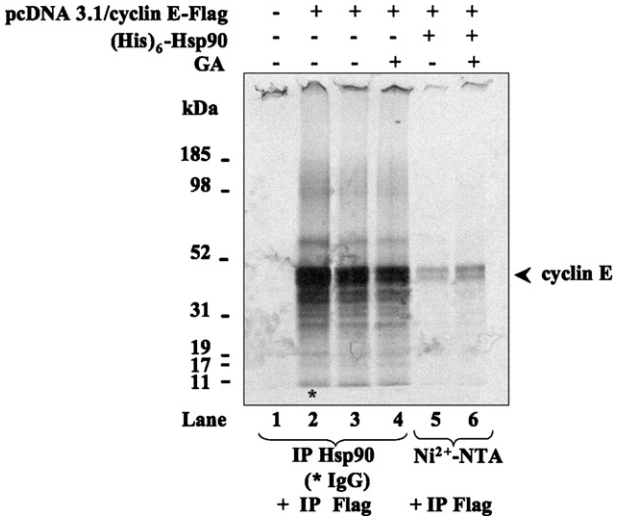

Fig. 5 – Newly synthesized cyclin E does not associate with Hsp90. In vitro transduction/translation of pcDNA3.1 cyclin E-FLAG construct was performed using the TNT Quick coupled reticulocyte lysate. The reaction media (50 μl) comprise 40 μl TNT Mix, 42 μCi [^{35}S]Methionine and 1 μg pcDNA3.1 cyclin E-FLAG, 57 nM $(His)_6$-Hsp90 and/or 500 nM GA as indicated. First, Hsp90 was immunoprecipitated with anti-Hsp90 (lanes 1, 3 and 4), with non-immune rabbit IgG (lane 2, indicated by *) or captured with Ni^{2+}-NTA agarose (lanes 5 and 6). A second round of immunoprecipitation was with anti-Flag antibody for all lanes. Samples were resolved by SDS-PAGE and [^{35}S]Methionine labeled proteins were revealed by exposing the dried gels on Kodak X-OMAT films overnight at −80 °C. Wedge indicates the position of cyclin E.

3.5. *Possible involvement of a PPIase in cyclin E destabilization*

Hsp90 forms heterocomplexes with various cochaperone proteins. Certain of these belong to the family of peptidyl-prolyl *cis–trans* isomerases (PPIases), one of which (Pin1) regulates the activities of several proteins involved in cell cycle progression, including cyclin E [36]. We considered the possibility that a PPIase may mediate the destabilization of cyclin E in cells exposed to GA. To address this point, we analyzed the effect of GA on two other proteins shown to be modulated by Pin1, cyclin D1 [37,38] and β-catenin [39,40]. HT29 cells were synchronized by aphidicolin and treated with GA as in Fig. 3, and the cell lysates were analyzed for the contents of these two proteins (Fig. 6). The results showed a marked decrease of cyclin D1 as a function of time of incubation with GA, whereas there was no effect on β-catenin. As for cyclin E, the effect of GA on cyclin D1 was countered by MG132. As cyclin D1 is not known to be an Hsp90 client protein, it is possible that its stability is regulated by Hsp90 in the same indirect manner as for cyclin E. We also searched for complexes between cyclin E and Pin1 by immunoprecipitation. These experiments gave negative results (data not shown).

4. Discussion

Cyclin E accumulation at the G1/S boundary promotes events that commit the cells to initiate DNA replication (S phase). Tight regulation of this process is a condition for normal cell proliferation [41–46]. Cyclin E overexpression resulting in high levels of the protein and deregulation of the Cdk2 kinase activity [47] has been observed in many human tumors [48,49]. This overexpression may trigger premature S phase entry and

genomic instability [50,51]. Moreover, high cyclin E levels correlate with aggressiveness of the tumor and poor prognosis [52,53].

We have previously shown that GA exerts an inhibitory effect on the cyclin E promoter in mouse fibroblasts and human cancer-derived cell lines [25]. In this study we show that GA acts not only at the transcriptional level but also on the cyclin E protein. In the HT-29 cells, the presence of GA induced a rapid decrease in the cyclin E level and this action was countered by the presence of CHX. These observations suggest that cyclin E is stabilized by Hsp90 and that its degradation requires the action of short-lived proteins, rapidly eliminated in the conditions where protein synthesis is blocked by CHX. In contrast, the cell content of cyclin A was not markedly affected by GA but its rapid degradation took place in cells exposed to CHX, indicating a short half-life of this protein. This result illustrates the selectivity of cyclin E destabilization by GA.

Many client proteins bind to Hsp90 and form multiprotein complexes in which they are maintained in a functional state and protected from degradation by the proteasome pathway [19,54]. GA inhibits the function of Hsp90 by competing with ATP for its binding site in the N-terminal domain [55,56]. In line with this pattern, we found that the inhibitor of the proteasome MG132 triggered the accumulation of ubiquitinylated forms of cyclin E as a function of time and this process was enhanced by the presence of GA. In order to verify the possibility that the protection of cyclin E by Hsp90 required the formation of a complex between these two proteins, we carried out experiments using immunoprecipitation as well as size-fractionation of cell lysates but failed to detect such complexes.

Several proteins form complexes with Hsp90 during or immediately after being released from the ribosome [35] (and references therein). This process is sensitive to GA but the complexes already formed can be resistant. This is for instance the case of Cdk2 whose elimination in cells exposed to GA reflects its turnover as newly synthesized Cdk2 cannot bind to Hsp90 and is destabilized [57]. We attempted to detect complexes between newly synthesized cyclin E and Hsp90 by using the coupled transcription/translation methodology, again without success.

Assuming that Hsp90 is the only direct target of GA it appears from our work that the protective effect of Hsp90 toward cyclin E is indirect: GA drives cyclin E toward ubiquitinylation and degradation by acting on (a) short-lived protein(s) which depend on Hsp90 for their activity or stability. The observation of such an indirect mechanism shows that Hsp90 can regulate the degradation of cellular proteins without binding to them. This conclusion suggests the existence of a novel category of proteins that are affected by the inactivation of Hsp90.

It has been shown that the turnover of cyclin E is accelerated by the action of the peptidyl-prolyl isomerase Pin1 [36]. Pin1, the first parvulin discovered in the human [58], represents a particular mode of regulation due to its ability to *cis–trans* isomerize specific phosphorylated motifs, thus providing a means of controlling protein function [59–62]. Although other proteins with PPIase activity do form complexes with Hsp90, such as immunophilins CyP40, FKBP51 and FKBP52 [26], Pin1 is not known to be an Hsp90-interacting protein. However, it has been proposed that Pin1 could participate in the restoration of tau protein activity as a co-chaperone in a scaffolding Hsp90 complex which could be destabilized by Hsp90 inhibitors [63]. Isomerization by Pin1 affects other proteins besides cyclin E, and, in particular, stabilizes cyclin D1 as well as β-catenin (reviewed in [64]). If GA acted by altering the activity of Pin1 or another PPIase, this should be reflected in similar ways on the cell contents of cyclin D1 and β-catenin. Our results showed that cyclin D1 levels were strongly reduced in cells exposed to GA (contrary to what would be expected if PPIase activity was involved in its turnover in the same manner as for cyclin E) but β-catenin levels were not modified. Data obtained in other laboratories [65,66] are in agreement with our observations concerning the effects of GA on cyclin D1 and β-catenin. Altogether, these results do not support the hypothesis that the indirect action of Hsp90 on cyclin E is mediated by PPIases.

An important point to note is that the effect of GA on cyclin E, as seen in the HT-29 cells, was not observed in another cancer-derived cell line, DU-145. Several other teams have reported that the effects of Hsp90 inhibition are often cell line-specific [67] and that deregulations of cancer cells can result in different responses to GA [68]. Srethapakdi et al. [69] who studied the effect of herbimycin A (another antibiotic of the ansamycin family) on signaling proteins proposed that the differences between the responses of different cell lines were ruled by the availability of the Rb protein. In our earlier work [25] we found that the DU145 cells which express a mutated, non-functional Rb, were still sensitive to the growth-inhibitory effect of GA. The presence of a functional Rb protein may however be related to the active degradation of cyclin E following the inactivation of Hsp90 by GA as seen in the HT-29 cells. This additional effect of GA may enhance its antitumoral activity. An accurate definition of the changes occurring in biochemical parameters such as the levels of cyclins could be useful for the early evaluation of the efficacy of different Hsp90 inhibitors as anticancer drugs, for instance in patients with accessible, inoperable tumors.

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