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Destabilization of Rb by human papillomavirus E7 is cell cycle dependent: E2-25K is involved in the proteolysis

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ABSTRACT

The HPV oncoprotein E7 promotes proteasomal degradation of the tumor suppressor protein Rb. In this study, we analyzed the regulation of E7-induced Rb proteolysis in HPV-containing Caski cervical cancer cells. We show that the Rb proteolysis is cell cycle dependent; in S phase Rb is stable while in post-mitotic early G1 phase cells and in differentiated cells, Rb is unstable. Similarly, the in vivo Rb/E7 interaction is not detected in S-phase cells, but is readily detected in differentiating Caski cells. The ubiquitinating enzymes involved in Rb proteolysis have not been identified. We find that the E3 ligase MDM2 is not involved in the Rb proteolysis in Caski cells. An in vivo analysis using multiple catalytic site mutant dominant negative E2 enzymes show that the C92A E2-25K most effectively blocks E7-induced Rb proteolysis. Taken together, these results show that E7 induces Rb proteolysis in growth-arrested cells and E2-25K is involved in the proteolysis.

Introduction

The retinoblastoma tumor suppressor protein Rb is a major regulator of multiple cellular processes including cell cycle, differentiation and apoptosis (Cobrinik, 2005; Dyson, 1998). The functions of Rb are impaired in majority of cancers by different mechanisms. Among them, increased phosphorylation and induced proteasomal degradation are frequent events. The function of Rb is regulated by phosphorylation through a cascade of cell cycle dependent kinases, and the molecular mechanisms have been studied extensively (Cobrinik, 2005). Others and us have shown that the E7 oncoprotein encoded by high-risk (HR) HPVs targets Rb for proteasomal degradation (Berezutskaya et al., 1997; Boyer et al., 1996; Jones et al., 1997). HR-HPVs are the etiological agents of cervical cancer and are associated with a subset of oral and head and neck cancer (Forastiere et al., 2001; Psyrri and DiMaio, 2008; zur Hausen, 2002). Oncogenic E7 binds Rb with a high affinity and the transforming activity of E7 depends on its ability to interact with Rb (Helt and Galloway, 2003). In non-HPV cells, the half-life of Rb is more than 6 h; however, in HPV-containing Caski and HeLa cervical carcinoma cells, the half-life of Rb is reduced to 2–3 h (Munger et al., 2004). Recently, multiple viral oncoprotein proteins including human cytomegalovirus (CMV) pp71 protein (Kalejta et al., 2003), the Epstein-Barr virus (EBV) nuclear antigen ENNA3C (Knight et al., 2005), the hepatitis C virus N55B (Munakata et al., 2005), and the HTLV-1 Tax oncoprotein (Kehn et al., 2005) were shown to induce proteasomal degradation of Rb. These studies show that although Rb is a stable protein with a long half-life, degradation of Rb by the proteasome may be a common pathway to overcome the growth inhibitory function of Rb during virus-induced tumorigenesis. Besides these viral proteins, two cellular oncoproteins, MDM2 and gankyrin, also destabilize Rb using the 26S proteasome (Higashitsuji et al., 2000; Sdek et al., 2005; Uchida et al., 2005). However, the mechanism of the proteasome-mediated degradation of Rb largely remained unknown.

Polyubiquitinated Rb accumulates in MG132-treated Caski cells (Wang et al., 2001). However, the enzymes involved in the polyubiquitination of Rb are unknown. Polyubiquitination is catalyzed by an enzymatic cascade involving the ubiquitin activating enzyme E1, the ubiquitin conjugating enzyme E2, and the ubiquitin ligase E3 (Hershko and Ciechanover, 1998). The E1 activates ubiquitin by ATP hydrolysis forming a thiol ester linkage with the c-terminus of ubiquitin. The E1–Ub conjugate then binds to an E2 and transfer the Ub to the E2. The E2s have the UBC domain that carries a conserved Cys residue involved in catalyzing ubiquitin conjugation (Jentsch, 1992; Pickart, 2001). The E3 ligases containing the ring finger or the hect domain often bind to a specific E2 and the substrate, and facilitate the transfer of ubiquitin from the E2 to the substrate. There is one ubiquitin activating enzyme E1, and studies by Boyer et al. (1996)

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showed that E7 cannot induce proteasomal degradation of Rb in cells containing a mutant E1. The E2 enzyme involved in the Rb proteolysis has not been identified. Two E3 ligases, MDM2 and APC, have been shown to interact with Rb (Binné et al., 2007; Sdek et al., 2005; Uchida et al., 2005); among them, only MDM2 can induce degradation (Sdek et al., 2005; Uchida et al., 2005). One study reported that the Rb proteolysis by MDM2 is proteasome dependent but Ub-independent (Sdek et al., 2005), while another suggested involvement of both components (Uchida et al., 2005). APC interacts with Rb but is not involved in Rb proteolysis (Binné et al., 2007). The cellular level of E7 is also regulated by the proteasome-mediated degradation (Gonzalez et al., 2001; Reinstein et al., 2000; Wang et al., 2001). E7 interacts with the Cullin1/Skp2, a cullin family of E3 ligase for its own ubiquitination (Oh et al., 2004). Rb also interacts with Skp2; however, the Skp2/Rb interaction is not involved in ubiquitination or proteasomal degradation of Rb (Ji et al., 2004). A recent study identified Cullin2 as an E7 binding protein, and suggested Cullin2 as an ubiquitin ligase for Rb proteolysis (Huh et al., 2007). However, the Cullin2–E7 interaction was observed only with the HPV16 E7.

In this study, we analyzed the cell cycle regulation of the proteasomal degradation of Rb in HPV-containing Caski cells. We observed that in S phase, Rb is mostly stable, whereas in post-mitotic early G1 phase cells or in differentiated cells, Rb is unstable. We showed that the E3 ligase MDM2 is not involved in the E7-induced proteolysis of Rb. We showed that C92A E2-25K, the catalytic site dominant negative mutant E2 efficiently blocked proteolysis of Rb by E7 suggesting that the ubiquitin carrier protein E2-25K supports E7-induced proteolysis of Rb.

Results

Proteasomal degradation of Rb is cell cycle dependent

The tumor suppressor protein Rb is active in early G1 phase of the cell cycle. Hypophosphorylated Rb associates with the transcription factor E2F to arrest cells in G1 phase. During G1 to S progression, and in S phase, Rb is hyper-phosphorylated by cyclin D-, cyclin E-, and cyclin A-dependent kinases, releases E2F, and becomes inactive as a growth suppressor (Cobrinik, 2005; Dyson, 1998). The HPVE7 oncoprotein induces proteasomal degradation of Rb (Berezutskaya et al., 1997; Boyer et al., 1996; Jones et al., 1997). In this report, we investigated the regulation of the proteolysis of Rb during cell cycle changes in HPV-containing cervical carcinoma cells. The half-life of Rb protein was determined by treatment with cycloheximide (25 μg/ml), and was found to be between 2 and 3 h in asynchronously growing Caski cells (Fig. 1A). For analyzing the half-life of Rb in S phase, the Caski cells were arrested by double-thymidine block, and more than 60% of the thymidine-arrested cells were in S phase (Fig. 1E). The S-phase enriched cells were treated with cycloheximide (25 μg/ml), and no significant decrease in the level of Rb protein was noticed for up to 4 h, suggesting that Rb protein is more stable in S phase (Figs. 1B and D). The half-life of Rb was found to be more than 6 h in S-phase enriched Caski cells (not shown). The thymidine-arrested cells were released from the S-phase block by growing in fresh medium, and the stability of the Rb protein was retained as cells progressed to G2 phase (Figs. 1C and D). The thymidine-released cell population was enriched in G2 phase (from 10.7% to 26.6%). The half-life of E7 protein did not change significantly during the S and G2 phase progression (Fig. 1). The HPV oncoprotein E6 induces degradation of p53 by the 26S proteasome; however, no significant change in p53 half-life was noticed in different cell cycle extracts of Caski cells (data not shown).

Enhanced proteolysis of Rb in post-mitotic cells during M to G1 progression

HPV-expressing carcinoma cells do not arrest in early G1 by serum starvation. Therefore, for analyzing the proteolysis of Rb in early G1 phase, the Caski cells were first blocked at M phase by growing in nocodazole (100 nM) for 20 h, and were then released from the mitosis block by growing in fresh medium for 4–6 h. Both mitotic and post-mitotic G1-enriched cells were treated with MG132. In mitotic cells, MG132 treatment only moderately increased the level of Rb. However, in post-mitotic cells, a dramatic decrease in the level of Rb was observed as the cells progressed to the G1 phase, and MG132 treatment efficiently restored the Rb level (Fig. 2A). Besides proteasome, caspases can also induce proteolysis of Rb (Dou and An, 1998). However, treatment of the post-mitotic cells with the caspase 3 inhibitor did not restore the Rb level. Therefore, the enhanced decrease in the level of Rb during the M to G1 phase progression in Caski cells was primarily due to proteolysis by the 26S proteasome. To further confirm enhanced proteolysis of Rb in post-mitotic cells, the HPV18-containing HeLa cells were arrested at M phase with nocodazole and released from M phase by growing in fresh medium. Both mitotic and post-mitotic HeLa cells were treated with MG132 for 4 h. Enhanced proteasomal degradation of Rb was also observed in post-mitotic Hela cells, and MG132 treatment restored the level of Rb (Fig. 2B). We analyzed the half-life of Rb in both mitotic and post-mitotic Caski cells. In post-mitotic extracts, the half-life of Rb decreased to less than 2 h in comparison to Rb half-life of 4 h in mitotic cells (Fig. 2C). Interestingly, we observed that the Rb proteins of different migrations have different half-lives in mitotic cell extracts (Fig. 2C). In mitotic cells, Rb is hyper-phosphorylated by multiple cyclin-dependent kinases, and during post-mitotic G1 progression, Rb is actively dephosphorylated with phosphatases to the active hypophosphorylated forms (Dyson, 1998). For further analysis, the mitotic cell extracts were probed with different phospho-specific Rb antibodies, and the different phosphorylated forms of Rb showed different decay rates (Fig. 2D). Interestingly, in the mitotic cells, the half-lives of Rb phosphorylated at the cyclin D-dependent phosphorylation site of T826 and S249/T252 were between 2 and 3 h, while the half-life of Rb phosphorylated at cyclin A/cyclin E dependent phosphorylation site of S612 was more than 4 h (Fig. 2D). A similar pattern of decay of the phospho-Rb species was also observed in asynchronous cells (not shown).

Enhanced proteolysis of Rb in differentiated Caski cells

Papillomaviruses replicate exclusively in differentiated epithelial cells (Stubenrauch and Laimins, 1999). One of the major biochemical functions of E7 is to keep the differentiated epithelial cells replication-competent to allow viral DNA replication (Cheng et al., 1995; Flores et al., 2000). Previous studies by other and us showed that high level of E7 is expressed in differentiated cells, and the half-life of E7 did not change significantly during differentiation (Cheng et al., 1995; Oh et al., 2006). For analyzing the half-life of Rb in differentiating cells, the Caski cells were grown in semi-suspension condition in medium containing 1.6% methylcellulose for 14 h (Reusch et al., 1998). Incubation of Caski cells for 14 h in the semisolid medium induces growth arrest and differential expression of keratin linked with differentiation (Oh et al., 2006). The Rb proteolysis was enhanced in differentiating cells, and the half-life of Rb was reduced to less than 2 h (Fig. 3B). As a control, the half-life of E2F-4, the major E2F in Caski cells, did not change significantly during differentiation.

Binding to E7 is critical for the proteasomal degradation of Rb (Berezutskaya et al., 1997; Dick and Dyson, 2002; Gonzalez et al., 2001; Jones et al., 1997). Detailed mutation analysis revealed that several single amino acid mutants of Rb, which are impaired in binding to E7, are spared from proteolysis (Dick and Dyson, 2002). To determine whether the cell cycle dependent proteolysis of Rb is due to lack of interaction, we compared the endogenous interaction between Rb and E7 in differentiated and S-phase specific extracts of Caski cells. The cell lysates were immunoprecipitated with antibodies against E7 and probed for both E7 and Rb protein using the Western blot assay.