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Review Article

The association of mammalian DREAM complex and HPV16 E7 proteins

Nurshamimi Nor Rashid, Hussin A Rothan, Mohd Shahrizal Mohd Yusoff

Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

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Abstract: The mammalian DREAM (*Drosophila*, RB, E2F, and Myb) complex was discovered in 2004 by several research groups. It was initially identified in *Drosophila* followed by *Caenorhaditis elegans* and later in mammalian cells. The composition of DREAM is temporally regulated during cell cycle; being associated with E2F-4 and either p107 or p130 in G0/G1 (repressive DREAM complexes) and with B-myb transcription factor in S/G2 (activator DREAM complex). High risk human papillomavirus (HPV) E6 and E7 oncoproteins expression are important for malignant transformation of cervical cancer cells. In particular, the E7 of high risk HPV binds to pRB family members (pRB, p107 and p130) for degradation. It has recently been discovered that the p107 and p130 'pocket proteins' are members of mammalian DREAM complexes. With this understanding, we would like to hypothesise the mammalian DREAM complex could plays a critical role for malignant transformation in cervical cancer cells.

Keywords: Human papillomavirus 16, E7 proteins, CaSki cell lines, mammalian DREAM complex

Introduction

HPV16 E7 targets pRB family members for degradation. The ability of HPV16 E7 to target pRB for degradation is necessary for malignant transformation [1]. In contrast to HPV16 E7, HPV6 E7 is not transforming and does not affect the stability of pRB or p107 [2, 3]; however it does target p130 for degradation [3]. pRB family members play a key role in regulating progression through the cell cycle. p130 is specifically up-regulated in G0/G1 and is responsible for keeping cells in a differentiated state [4]. The fact that both high risk and low risk E7 target p130 for degradation may indicate that p130 is important for the HPV life cycle. Targeting p130 for degradation may be conducive to creating an 'S phase-like' environment [5-7].

Mammalian DREAM complex

A multiprotein subunit has been identified in humans which is involved in cell cycle regulation. This complex is known as the DREAM or LINC complex. It was originally discovered in *Drosophila melanogaster*, in which it is involved in transcriptional repression [8, 9]. The complex is known as dREAM (Drosophila, RB, E2F and Myb-interacting proteins) [8] or Myb-MuvB [9] (**Table 1**). In addition to RB/E2F this complex also contains a Drosophila MYB transcription factor, three Myb-interacting proteins (Mip40, Mip120 and Mip130) and a protein related to the mammalian pRB-binding protein RbAp48. It is also suggested that dREAM/Myb-MuvB complexes are highly conserved in evolution since they are related to the *Caenorhabditis elegans* synMuv class B genes, except dMYB. The synMuv class B proteins form a complex which is known as DRM [10]. The homologs of all subunits of the invertebrate complexes have also been identified in human complexes, named DREAM or LINC, whose composition is regulated at distinct phases of the cell cycle [11-15]. The core DREAM complex contains Lin9, Lin37, Lin54, Lin52 and RbAp48 (the human homologues of Drosophila Mip130, Mip40, Mip120, dLin52 and Caf1p55, respectively). The pRB family members, p130 and p107 were comprised in human DREAM complex as a transcriptionally repressive during the course of a cell cycle. The composition of DREAM is temporally regulated during the cell cycle, being associated with E2F-4 and either p107 or p130 in

Table 1. Comparison of dREAM (*Drosophila melanogaster*), DRM (*Caenorhabditis elegans)* and DREAM/LINC (human) complexes constituents

Drosophila melanogaster		*Caenorhabditis elegans*	Humans	
dREAM	MMB	DRM	DREAM/LINC	
RBF1, RBF2	RBF1, RBF2	LIN-35	p130, p107	-
dE2F2	dE2F2	EFL-1	E2F4/E2F5	-
dDP	dDP	DPL-1	DP1/DP2	-
P55/CAF1	P55/CAF1	LIN-53	RBBP4	RBBP4
MYB	MYB	-	-	B-MYB
MIP130	MIP130	LIN-9	LIN-9	LIN-9
MIP120	MIP120	LIN-54	LIN-54	LIN-54
MIP40	MIP40	LIN-37	LIN-37	LIN-37
-	LIN-52	LIN-52	LIN-52	LIN-52
-	L(3)MBT	-	-	-
-	RPD3	-	-	-

DREAM was first discovered in *Drosophila melanogaster*embryonal cells. *Drosophila* dREAM complex is resistant to dissociation by CDK-phosphorylation and they exist throughout cell cycle. The dREAM/MMB complexes are also highly conserved in evolution since they are related to the *Caenorhabditis elegans* (DRM). The homologs of all subunits of the DRM complexes have also identified in human complexes, named DREAM/LINC, whose composition is regulated at distinct phases of the cell cycle. Adapted from [74].

G0/G1 [12, 13, 15, 16] and with the B-myb transcription factor in S/G2 [12, 13, 15, 16].

Although the DREAM complex is closely related to the DRM and dREAM/Myb-MuvB complexes, pocket proteins, B-myb and E2F transcription factor do not form part of the stable core complex. The complex dynamically interacts with pocket proteins/E2F-4 or B-myb in a cell cycle-dependent manner [15]. During quiescence, DREAM is present on the promoters of E2F-regulated genes required for G1/S and G2/M progression in complex with p130 and E2F4 [13, 17]. During cell cycle re-entry, the promoter specificity of the DREAM complex changes. In late G1, DREAM/p130/E2F4 complexes dissociate from the promoters of genes required for G1/S progression. This allows the activator E2F (1-3) transcription factors access to promoter and results in the expression of genes required to drive the cell through G1/S. On promoters of genes required for G2/M progression, DREAM selectively interacts with B-myb during S/G2. RNAi studies have shown that DREAM and B-myb co-activate a specific cluster of genes required for G2/M phase. These include cyclin B1, cyclin A2 and cdc2, which are required for G2/M progression, BUB-1 and CenPE, which are required at the mitotic spindle checkpoint, Aurora kinase-A and Plk-1, which are required for spindle assembly and UbCh10, which is required for exit from mitosis [13, 17]. This review will focus mainly on the relationship and mechanisms between the DREAM complex and HPV16 E7 proteins.

HPV16 E7 disrupts p130/DREAM complex

Several observations have provided evidence for the disruption of p130/DREAM complexes and p107/DREAM complexes in HPV16 E7 positive cells (Caski and SiHa) [18]. In both SiHa and CaSki cells, the lowering of p130 levels was shown by Western blot and is presumably due to E7-mediated degradation [3]. HPV16 E7 is able to induce the proteasomal degradation of p130 and the related pocket proteins in keratinocytes and this is a distinct function of the HPV16 E7 protein that is not shared by adenovirus E1A or SV40T antigen [19]. The proteasome is a large 26S multisubunit complex that degrades polyubiquitylated proteins to small peptides. Proteasomes act on proteins marked specifically for degradation by a small protein called ubiquitin [20]. Ubiquitin is activated for transfer to substrate through the ATP-dependent formation of a thioester bond with the ubiquitin-activating (E1) enzyme and is subsequently transferred to a ubiquitin-conjugating (E2) enzyme. Finally, thioesterified ubiquitin is transferred to the target protein with the assistance of a ubiquitin ligase (E3). E3s bind directly to substrate, suggesting that they provide specificity in ubiquitylation reactions. SCF complexes (E3 ubiquitin ligases) recognize and polyubiquitylate substrates in a phosphorylation-dependent manner, targeting them for degradation by the 26S proteasome [21]. HPV16 E7 and p130 both interact with and are ubiquitylated by SCFSkp2 complex [22, 23].

Nor Rashid et al. also showed that p130/DREAM complex was disrupted, particularly in CaSki when compared to T98G cells in which of the p130/DREAM complex was expressed

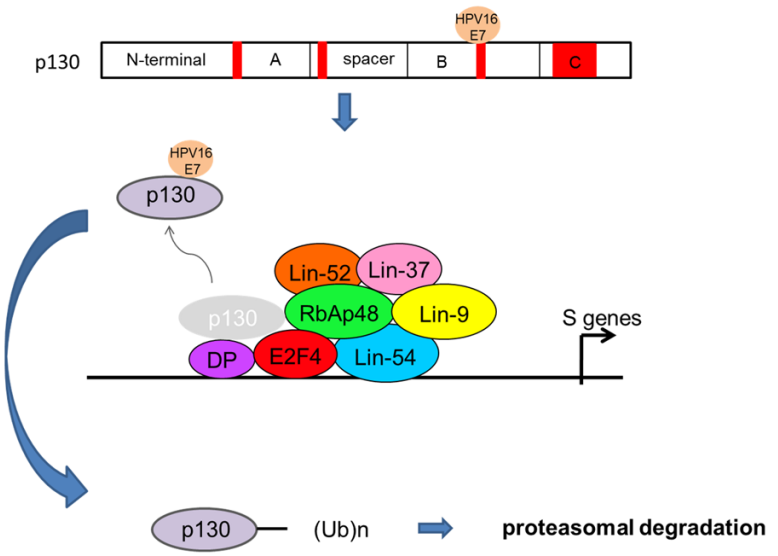

Figure 1. HPV16 E7 oncoproteins binding to p130-DREAM complex through their L-X-C-X-E motif resulting in proteasomal degradation. The p130 B pocket was critical for binding L-X-C-X-E (leucine-X-cysteine-X-glutamate) (X denotes any amino acid residue) motif containing proteins including HPV16 E7. The HPV16 E7 targets p130 at the B pocket, resulted in the proteasomal degradation, in which the p130 proteins are degraded by the ubuiquitin-proteasome pathway. Both, HPV16 E7 and p130 interact with and are ubiquitilated by SCF^{Skp2} [22, 23].

abundantly. This presumably reflects the binding of p130 to E2F4 by 16E7. Both HR (HPV16 E7) and LR (HPV11 E7) proteins bind pRB family members through their LXCXE binding motif [24] (**Figure 1**). Furthermore, several *in vitro* studies have revealed that HPV16 E7, in contrast to HPV6 E7, has a greater affinity for pRB, p107, and p130 [25, 26]. HR HPVs destabilize all pRB family members and this is a critical event that drives cellular transformation [19, 27-31]. The main contributing factor that results in enhanced binding of HR HPV E7 to pRB and its ability to target pRB for degradation is an aspartic acid versus glycine residue in HR vs. LR E7 proteins at the amino acid immediately before the LXCXE binding motif. Although HPV6 E7 has a lower affinity for binding p130 than HPV16 E7, it is as efficient in targeting p130 for degradation [3]. The E7 proteins from the low risk HPV types bind to the pocket proteins with lower affinity than the high risk HPV E7 types.

B-myb is over-expressed in 16E7-containing cells, as the G0/G1 transcriptional repression (presumably mediated by p130/DREAM complex) is relieved [18, 32]. This is due to the inactivation of pRB family proteins by 16E7 protein which subsequently causes the G1 exit and cell cycle entry to S-phase. On the other hand, the higher expression of B-myb/DREAM complexes might be related to the interaction of 16E7 protein with the cyclin A/CDK2 complex [33] which will ensure the cells will remain in an S-phase like state where B-myb gene expression is maximal. Nor Rashid et al. also found that p130/DREAM complex was abundant in T98G cells (control cell line). Claudio et al. has demonstrated that in certain cell lines, such as T98G cells, which are deficient in the CDK inhibitor, p16, the p130 protein is the major cell cycle inhibitor instead of pRB and p107. However, in the C33A cell line there was a higher expression of p107 as indicated in the input control. This may be related to the lack of pRB as a cell cycle inhibitor in C33A cells [34].

Re-expression of p53 tumour suppressor protein in E7 depleted-CaSki cells

Depletion of E7 in CaSki cells inevitably suppressed E6 oncoproteins in this cell lines since all HPVs have a conserved structure of the early promoter, the E6 promoter or p97 including HPV16, that initiates transcription of the E6/E7 polycistronic mRNA. Therefore, p53 was re-expressed upon E6 depletion which could also impact on DREAM complexes. Under these conditions, $p21^{Cip1}$ could inhibit cyclin E/A cdk2 complexes, which would preclude both activating phosphorylation of B-myb [35] and inactivating phosphorylation of p130 [36].

p53 plays many roles in cell-cycle regulation. It activates repair proteins in response to DNA damage, and if this damage is irreparable can induce cell arrest by activating p21, a cyclin kinase inhibitor [37, 38]. High risk HPV E6 binds to E6-associated protein (E6-AP), a cellular ubiquitin-ligase, and targets the tumour suppressor p53 for degradation [39, 40]. Activation of p53 occurs upon cellular stresses, such as

DNA damage, oncogene activation, telomere erosion and hypoxia. It is mediated, at least in part, by inhibition of MDM2 and rapid stabilization of the p53 protein by post-translational modifications. E6 protein from the high -risk HPV type 16, has a higher affinity in binding towards p53 and by binding and targeting p53 for degradation, it prevents cell cycle arrest and apoptosis in stressed cells favouring accumulation of DNA damages and cellular transformation [41]. Both viral oncoproteins (E6 and E7) are able to form stable complexes with cellular proteins and alter, or completely neutralise, their normal functions. These events lead to the loss of control of cell cycle check points, of apoptosis and differentiation and eventually to transformation of the HPV infected cell. As hypothesised, the p53 expression was increased upon E7 suppression in CaSki cells [18].

Defective in the B pocket of p130 abrogate the HPV-transformed cells proliferation

HPV16 E7 binds to a highly conserved shallow groove on the B pocket of hypophosphorylated pRb, an interaction that is mediated by the LXCXE motif [42, 43]. The CR2 region contains a conserved LXCXE sequence which interacts with the retinoblastoma tumor suppressor protein pRB and the related 'pocket proteins' p107 and p130 [24, 44]. These proteins interact with the transcription factor E2F, which is able to regulate cell cycle transition [45-47]. The primary activity of high risk E7 proteins is to inhibit members of the retinoblastoma (RB) tumor repressor family to induce progression into S phase [48]. In normal cells, pRB is hypophosphorylated in early G_1 and becomes increasingly phosphorylated towards S phase. In its hyphophosphorylated form, RB binds E2F transcription factors and actively represses transcription from promoters containing E2F sites. By binding pRB in a hypophosphorylated state, E7 prevents it from binding to E2F and thereby promotes cell cycle progression [45]. In normal epithelia, cell cycle exit occurs following differentiation. By binding to pRB, E7 promotes cell cycle progression in differentiated epithelial cells, allowing for replication of the HPV genome.

p130 localization

p130 contains three nuclear localization signals (NLS), two in the C-terminus and one in the loop region [49]. In undifferentiated cells, hypophosphorylated p130 is predominantly in the nucleus in the G0/G1 phase of the cell cycle. In S-phase, p130 is typically phosphorylated and transported to the cytoplasm where it is targeted for degradation. Shuttling of p130 between the nucleus and the cytoplasm therefore provides a means of regulation [23, 49]. p130 levels, like the levels of other pRB family members, are regulated in response to the proliferative state of cells and are controlled by Skp-Cullin-F-box (SCF) complexes which mediate proteolysis in a phosphorylation-dependent manner [23, 50-52]. p130 has been shown to be phosphorylated in cycling cells by cyclin D/Cdk4 or Cdk6, cyclin A/Cdk2 and cyclin E/Cdk2 [4, 50]. Cdk4/Cdk6, not Cdk2, is responsible for targeting p130 for degradation in fibroblasts [23]. In cycling cells Cdk4/Cdk6 phosphorylates p130 on Ser 672, resulting in a hyperphosphorylated form of p130 that is targeted for degradation by an SCF 21 complex [23]. In growth-arrested and terminally differentiated cells, p130 is phosphorylated by glycogen synthase kinase 3 (GSK3) in the loop region in the B subdomain and thus stabilized [53].

Disruption of the pRB/Lin-9 interaction by the E7 proteins

The Lin-9 protein is known to associate with pRB in mammalian cells, a function that appears to mediate a number of its known activities [54]. As hLin-9 mediates pRB tumour suppressor activity through an interaction with the pocket domain, it is interesting to speculate as to whether the E7 proteins could antagonise this interaction. The high risk HPV16 E7 protein contains a LXCXE domain that is known to bind to the pocket domain of pRB and suppress its tumour suppressor activity. It is possible that oncoproteins such as the HPV16 E7 protein may compete for binding with the hLin-9 protein, thus diminishing its functional activity.

The strength of binding to pRB varies between different HPV types. Although generally speaking, the low risk and cutaneous HPV E7 proteins do not bind to pRb with a particularly high affinity and the high risk HPV E7 proteins do [55], there is a grey area containing many in-between [25, 56, 57]. In contrast to the high risk HPV types, low risk and cutaneous E7 proteins contain either a fully intact LXCXE motif, a partial LXCXE motif or no LXCXE motif [56]. It is unclear why E7 proteins such as the cutaneous HPV1

E7 protein, which contains an intact LXCXE motif and is able to bind to pRb with a similar affinity to the high risk HPV16 E7 protein, does not have any *in vitro* transforming activity [25]. It is thought that the ability to target pRB for proteasomal degradation plays an important role, as the HPV1 E7 protein is not able to degrade pRB [19, 58, 59], but this is not sufficient for cellular transformation [60]. The additional functional activities of the high risk HPV E7 proteins that enable the proteins to efficiently overcome the G_1/S checkpoint and promote cellular transformation have not yet been fully characterised, but are thought to include the ability to inactivate the CDKi $p21^{WAF1/CIP1}$ and induce genomic instability. Presumably the functional amalgamation of each of these activities defines the relative efficiency by which each E7 type may deregulate the cell cycle.

It would be interesting to see whether the affinity of the E7 type for pRB is directly comparable with an ability to antagonize pRB-hLin-9 binding, and whether this has any functional relevance. It is possible that the efficiency by which an E7 type disrupts the pRB-hLin-9 interaction is not just determined by its relative affinity for pRB. The cutaneous HPV1 E7 protein for example, may not be able to antagonize the pRB-hLin-9 interaction as efficiently as the high risk HPV16 E7 protein. In such a situation, the ability to prevent pRB-hLin-9 binding would be dependent on other unidentified activities or functional domains of the particular E7 type. If this were the case, it could mirror the disruption of the pRB-E2F interaction. Although the LXCXE motif is required to anchor the HPV16 E7 protein to pRB, an additional C-terminal region is required to disrupt E2F binding [61]. By equal measure, it is possible that the efficiency by which an E7 type disrupts the pRB-hLin-9 interaction does not determine the functional activity of the pRB-hLin-9 complex. The HPV1 E7 protein may be able to stoichiometrically disrupt the pRB-hLin-9 complex in a similar manner to the HPV16 E7 protein, but this may not have the same functional effect. For example, as the HPV1 E7 protein is not able to degrade pRB, the E7 proteins may quickly become saturated.

Since the hLin-9 protein synergises with pRB in the activation of genes required for differentiation, a situation may be envisaged whereby the high risk E7 proteins could prevent the expression of these genes. If this scenario were true, high risk E7 proteins could simultaneously drive the cell through G_1/S and prevent the expression of genes required for terminal differentiation. This would result in a pool of continually dividing, undifferentiated cells similar to those seen in high grade infections [62].

B-myb/DREAM as an activating complex in cell cycle progression

Avian myeloblastosis viral oncogene homolog-2 (*mybl2*) is a member of a multigene family of transcription factors involved in control of cell cycle progression, differentiation and apoptosis [22, 63]. All members of this family, A-Myb, B-Myb (MYBL2) and C-Myb, contain conserved regulatory and transactivation domains that exhibit sequence-specific DNA-binding activity. Only B-Myb, the ancestral gene of this family, is expressed in all proliferating cells [64].

As with many cell cycle associated transcription factors, B-Myb expression and function is dynamically regulated. The *mybl2* gene, which encodes B-Myb, is regulated directly by E2F transcription factors and is maximally induced at the G1/S boundary of the cell cycle [63]. The *trans*-activation and gene regulatory potential of B-Myb is regulated by cyclin A/cdk2-mediated phosphorylation [65], and B-Myb is degraded through a ubiquitin-mediated process late in S phase [66]. In S/G2, the DREAM complex associates with B-Myb to activate genes required for G2/M transition and mitosis [15]. As demonstrated by Nor Rashid et al. B-Myb is abundantly expressed in CaSki cell line which is due to the inactivation of p130 pocket protein by HPV16 E7 protein which subsequently causes the G1 exit and cell cycle entry to S-phase. Pang et al. has shown that HPV16 E7 could bind to the B-Myb/DREAM/FoxM1 complex in the absence of pocket proteins family. FoxM1 is the transcriptional activator of mitotic genes and E2F target gene [67]. FoxM1 has also been implicated in cellular activities, including regulation of oxidative stress, repair of DNA damage and tumour metastasis [68, 69]. However, overexpression of FoxM1 has been reported in poor prognosis cervical cancer [70, 71]. Pang et al. has also reports that HPV E7 interacts with the core component of the DREAM complex, Lin-9, and with transcription factors B-Myb and FoxM1 to promote mitosis. They also

showed that the association between Lin-9 and FoxM1 depended on the LXCXE motif of E7, whereas E7 association with B-Myb did not. Moreover, the B-Myb/DREAM complex does not contain the p130 or any of the pocket protein family [72]. However, it is quite vague on how and where HPV16 E7 binds to B-Myb/DREAM/FoxM1 complex.

Conclusions and future perspectives

Taken together, it is necessary for HPV16 E7 protein to disrupt the p130/DREAM complex to promote cell cycle progression in cervical cancer cells. HPV16 E7 protein will interfere with p130/DREAM complex during G0/G1 to promote into S phase in which requires B-Myb/DREAM complex to be activated to express the genes which are required for S/G2/M phase. To date, there are two forms of DREAM complexes in mammalian cells, the repressor p130/DREAM complex and the activator B-Myb/DREAM complex. It should also be noted that some features of the DREAM complex remains poorly understood. In the context of RAS oncogene however, Tschop et al. has shown that p130/DREAM is important for establishment of senescence [73]. Further investigation on the relationship between HPV16 E7 and the mammalian DREAM complex is required and will provide more insight on cervical oncogenesis and viral regulations.

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Disclosure of conflict of interest

None.

Address correspondence to: Nurshamimi Nor Rashid, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia. Tel: +603-79677549; Fax: +603-79674954; E-mail: nurshamimi@um.edu.my

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