Orf virus cell cycle regulator, PACR, competes with subunit 11 of the anaphase promoting complex for incorporation into the complex

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The poxvirus anaphase promoting complex regulator (PACR) promotes viral replication by manipulating the anaphase promoting complex/cyclosome (APC/C), a multisubunit ubiquitin ligase complex with essential roles in cell cycle regulation. PACR has sequence similarities to APC/C subunit 11 (APC11) and associates with APC/C subunits. However, unlike APC11, expression of PACR disrupts APC/C functions. Here, we further investigated the interaction of PACR with APC/C. Following knockdown of APC1, the subunit linking APC11/APC2 to the rest of APC/C, PACR remained bound to APC2 but not to other, distal, subunits of the complex, suggesting PACR associates with APC/C via APC2. This was supported by the demonstration, in vitro, of a direct interaction between PACR and APC2. Moreover, the presence of PACR interfered with interactions between both APC11 and APC2. Based on these observations we propose that PACR competes with APC11 for the incorporation into APC/C.

The ubiquitin proteasome degradation pathway has critical roles in cell cycle progression. Key players in this system are cullin-RING ubiquitin ligase complexes, such as the anaphase promoting complex/cyclosome (APC/C). Human APC/C is composed of 12 subunits with a catalytic core consisting of APC11, a RING-H2 protein and APC2, a scaffold protein. APC/C targets a broad spectrum of substrates for proteasome degradation in an orderly coordinated fashion ensuring controlled cell cycle progression (van Leuken et al., 2008). Thus far only a small number of viruses have been shown to manipulate APC/C (Bellanger et al., 2005; Kim et al., 2008; Liu et al., 2009; Mu et al., 2010; Teodorog et al., 2004; Tran et al., 2008). Recently, we showed that PACR (poxvirus anaphase promoting complex regulator), a partial mimic of APC11, facilitates viral replication by disrupting APC/C function (Mo et al., 2009, 2010). PACR interacts with APC2 in a manner similar to APC11, but unlike APC11 the RING-H2 domain of PACR lacks ubiquitin ligase activity. Therefore, it seems that PACR might bind APC2 directly and interfere with APC11’s binding to APC2. Here, we test this possibility and show that PACR associates with APC/C through APC2, that it interferes with APC11’s binding to APC2 and that expression of PACR induces APC11 degradation through the 26S proteasome pathway.

APC11 binds directly to the C-terminal cullin homology domain (CHD) of APC2 (Tang et al., 2001). We have shown that PACR associates with the C-terminal region of APC2 when both proteins are co-expressed in cultured cells. It was not clear if this binding was direct or required the participation of a third party (Mo et al., 2009). These interactions were therefore examined in vitro using purified components. APC11-His and PACR-His were expressed in Escherichia coli and purified by the Ni-NTA affinity column as described previously (Mo et al., 2009). In addition, the N-terminal region of human cyclin B (NT-CycB, aa 1–87) carrying a C-terminal FLAG-6×His tag was expressed and purified in the same manner. The CHD of APC2 was expressed from a pET21D vector, such that its N terminus was tagged with glutathione S-transferase (GST). This protein (GST-APC2 CHD) was expressed in the E. coli strain BL21D and purified using Glutathione Sepharose 4B (17-0756-01; GE Healthcare) but was not eluted from the Sepharose beads. GST was prepared in the same way. APC11-His or PACR-His was mixed with GST-APC2 CHD Sepharose beads or GST Sepharose beads in wash buffer 1 (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM DTT and EDTA-free protease inhibitor; Roche). After incubation for 4 h at 4 °C, immunoprecipitates were washed four times (5 min each) in wash buffer 1 and three times (5 min each) in wash buffer 2 [20 mM Tris/HCl pH 7.5, 250 mM NaCl, 1 % (v/v) Triton X-100, 10 % (v/v) glycerol, 1 mM DTT and EDTA-free protease inhibitor; Roche]. Precipitated proteins were detected by SDS-PAGE and Western blotting. APC11 and PACR were captured by GST-APC2 CHD (Fig. 1a, lanes 1 and 3), but not by GST (Fig. 1a,
PACR, like APC11, binds APC2 CHD directly and inhibits interaction between APC11 and APC2 CHD. (a) PACR binds APC2 CHD directly. GST-APC2 CHD or GST were immobilized on the GST Sepharose beads (Glutathione Sepharose 4B) and incubated with either purified APC11-His or PACR-His at the concentration indicated. After incubation and subsequent washing, the proteins retained on beads (IP) were analysed by SDS-PAGE and Western blotting (WB). The top panels show signal detected when amounts equivalent to 10% of the input used in the binding assay were analysed by SDS-PAGE and WB. (b and c) PACR interferes with APC11 binding to APC2 CHD. Immobilized GST-APC2 CHD was mixed with APC11-His and NT-CycB-FLAG-His (b) or PACR-His (c) at the indicated concentration. After washing, APC11 remained bound to APC2 CHD and was detected by anti-APC11 WB. The intensity of APC11 signals were monitored using the Quantity One software (Bio-Rad). The ratio between APC11 remaining bound to APC2 CHD (residual APC11) and input APC11 (10%) is indicated.

Next, we tested whether PACR would compete with APC11 for binding to APC2 CHD. Immobilized GST-APC2 CHD was mixed with various concentrations of APC11-His alone (Supplementary Fig. S1, available in JGV Online) or with mixtures containing APC11-His and either a control protein, NT-CycB-FLAG-His (Fig. 1b) or PACR-His (Fig. 1c). After washing, any APC11 remaining bound to the GST-APC2 CHD beads was detected by anti-APC11 Western blot analysis. This revealed that PACR-His efficiently blocked binding of APC11 to APC2 CHD (Fig. 1c), but NT-CycB did not (Fig. 1b). Increasing the concentration of the non-competing control protein NT-CycB had little effect on the relative amount of APC11 that remained bound to APC2, with 7.8, 7.3 and 8.6% of the input APC11 detected (Fig. 1b, residual APC11). Similar results were obtained using only APC11 and APC2 (Supplementary Fig. S1). In contrast with the non-competing protein, the amount of APC11 remaining bound to APC2 dropped (6.2, 1.9 and 0.007%) as the concentration of PACR was increased (Fig. 1c residual APC11). These results showed that PACR-His specifically blocked binding of APC11 to APC2 CHD.

Co-precipitation assays have shown intracellular interaction of PACR with three subunits of APC/C (APC2, APC3 and APC4) (Mo et al., 2009). It remains unclear if this represents PACR binding to an essentially complete APC/C through binding to APC2 or independent binding to individual subunits. APC/C is composed of two subcomplexes one of which consists of APC11 and APC2, while the other includes APC3 and APC4 along with most of the other subunits. APC1 acts as a bridging unit that links the two subcomplexes (Fig. 2a) (Thornton et al., 2006). If PACR associates with the complex, rather than the separate subunits and does so through APC2, then deletion of APC1 would disrupt the association of PACR with subunits such as APC3 and APC4, but not APC2. 293T cells were transiently transfected with either APC11-FLAG or PACR-FLAG and transfected with or without short interfering RNA (siRNA) targeting APC1 (1299003; Invitrogen). Immunoprecipitates prepared with anti-FLAG agarose beads were analysed by Western blotting (Fig. 2b). Co-precipitation of APC4 and APC2 with either APC11 or PACR was readily detected in lysates.
prepared from cells that were not treated with siRNA-APC1 (Fig. 2b, lanes 1 and 3). In contrast, APC4 no longer precipitated with APC11 or PACR after APC1 was knocked down by siRNA against APC1 (siRNA-APC1), but precipitation of APC2 with either APC11 or PACR was unaffected by this treatment (Fig. 2b, lanes 2 and 4). When APC3 was immunoprecipitated in an independent experiment, the association of APC11-FLAG, PACR-FLAG or APC2 with APC3 was abolished by treatment with siRNA-APC1 (Fig. 2c, lanes 2 and 4), but not in untreated cells (Fig. 2c, lanes 1 and 3). Thus, knocking down the APC1 bridging unit resulted in dissociation of PACR, APC11 and APC2 from other subunits of the complex. This suggested that, like APC11, PACR associated with APC/C through binding to APC2.

The above data showed that PACR bound APC2 directly and in doing so competed with APC11. We next examined if PACR inhibited the binding of APC11 to APC2 in the intracellular environment. To address this question, endogenous APC2 was immunoprecipitated from 293T cells transiently transfected with either APC11-FLAG or PACR-FLAG plasmids and co-precipitated proteins were examined by Western blotting. As expected, APC11-FLAG and PACR-FLAG co-precipitated with APC2 (Fig. 3a, lanes 2 and 3). However, although endogenous APC11 was readily detected as co-precipitating with APC2 in the control reactions, it was hardly detectable when PACR was present (Fig. 3a, lane 3). In contrast, levels of APC4 and APC3 co-precipitating with APC2 were unaffected by the presence of PACR (Fig. 3a, lanes 2 and 3). Anti-cyclin A immunoprecipitation was used as a negative control. None of APC2, APC3, APC4, APC11 or PACR-FLAG co-precipitated with anti-cyclin A (Fig. 3a, lane 4). Therefore, PACR inhibited the binding of APC11 to endogenous APC2.

Previous studies have shown that binding of APC11 to APC2 protects APC11 from degradation by the 26S proteasome pathway and that when ectopically expressed APC11 is not bound by APC2 it is degraded promptly (Ohta et al., 1999). Therefore, a predicted consequence of inhibition of APC11 binding to APC2 by PACR was that free APC11 would be degraded. To examine this possibility, PACR-FLAG was transiently expressed in 293T cells and 4 h before harvesting, cells were treated with MG132 to inhibit the 26S proteasome pathway. Levels of endogenous proteins of interest were examined by Western blotting (Fig. 3b, c and d). Expression of PACR reduced the levels of APC11 detected but had no effect on APC3 or ROC1, a RING-H2 protein related to APC3. PACR competes with APC11 for binding to APC2.
The drop in APC11 levels was facilitated by the 26S proteasome pathway as the addition of MG132 stabilized APC11 (Fig. 3b, lane 4). We have shown previously that the N-terminal region of PACR is required for binding to APC2 and in this respect PACR mimics APC11 (Mo et al., 2009). Consistent with this, expression of a PACR construct lacking the N-terminal region (PACR-CT) failed to suppress APC11 levels (Fig. 3b, lanes 5 and 6). Furthermore, the reduction in APC11 levels showed a dose-dependent response to increasing amounts of PACR (Fig. 3c). In these experiments, we also examined the insoluble pellet of cell debris remaining after cell lysis and saw no evidence of precipitated APC11 upon PACR expression (Fig. 3d). Taken together, our data

**Fig. 3.** Transiently transfected PACR inhibits binding of endogenous APC11 to APC/C and promotes degradation of APC11 through the 26S proteasome pathway. (a) PACR interferes with incorporation of APC11 into APC/C. 293T cells were transfected with the indicated plasmids (+) and processed as described in Fig. 2. APC2 or cyclin A was precipitated (IP) using specific antibodies (ab18295; Abcam and Ab2; Calbiochem, respectively). After SDS-PAGE, proteins of interest were identified by WB as indicated. (b) PACR promotes APC11 degradation through the 26S proteasome pathway. 293T cells (4×10^5 cells in a 35 mm diameter dish) were transfected with 1 μg of the indicated plasmids (+). Cells were treated with (+) or without (−) 10 μM MG132 for 4 h before harvesting. Cell lysates were collected and proteins of interest were examined by WB. (c) PACR promotes proteasomal degradation of APC11 in a dose-dependent manner. 293T cells were transfected with the indicated amount (μg) of PACR or PACR CT plasmids with or without MG132 and processed as described in (b) before WB was performed to identify indicated proteins. (d) PACR does not precipitate APC11. 293T were transfected with indicated plasmids (+) and processed as described in (b). Lysates were separated into supernatant (S) or pellet (P) by centrifugation before WB to detect the indicated proteins.
indicate that PACR expression inhibited the binding of APC11 to APC2, leading to reduced levels of APC11.

The data presented here show that PACR binds APC2 directly and that PACR associates with APC/C through APC2. Furthermore, we showed that expression of PACR leads to reduced levels of APC11 through a pathway involving proteasome activity. A plausible functional model consistent with these observations is that PACR competes with APC11 for binding to APC2 and is incorporated into APC/C in place of APC11. This results in the proteasomal degradation of unbound APC11. We cannot rule out the fact that there might be additional ways by which PACR reduces APC11 levels. However, it is unlikely that PACR directly promotes APC11 ubiquitination and degradation because the PACR RING-H2 domain does not demonstrate ubiquitin ligase activity (Mo et al., 2009).

A consequence of PACR’s competition with APC11 might be the formation of an APC2/PACR subcomplex that did not bind the other components of APC/C. Our immunoprecipitation assays showed co-precipitation of endogenous APC3, APC4 and APC2 with transiently transfected PACR, suggesting that PACR is capable of associating with otherwise intact APC/C. However, the formation of an APC2/PACR subcomplex was seen in cells treated with siRNA-APC1 (Fig. 2b, lane 4). In addition, less APC4 appeared to precipitate with PACR than was seen with APC11, and less PACR than APC11 co-precipitated with APC3 (Fig. 2b and c, lanes 1 and 3). These observations suggest that APC11 displays a higher affinity for APC2 than does PACR and are consistent with our previous observations (Mo et al., 2009). This could be a specificity issue as, although orf virus infects humans it is regarded as a sheep virus and PACR might bind the human APC used here more weakly than sheep APC2. Nevertheless, it remains possible that PACR disrupts APC/C, in part, by sequestering APC2. Further investigations will be required to address this issue.

To date, six other viruses have been proposed to target APC/C. Three of these viruses activate APC/C. Hepatitis B virus activates APC/C by relieving APC/C of its endogenous inhibitor, human T cell leukemia virus type 1 activates APC/C by unknown means and adenovirus activates APC/C by modifying the phosphorylation status of APC/C (Kim et al., 2008; Liu et al., 2005; Mui et al., 2010). Strategies used by viruses to inhibit APC/C include interfering with the integrity of the complex [human cytomegalovirus (Tran et al., 2008), chicken anaemia virus (Heilman et al., 2006; Teodoro et al., 2004) and adenovirus (Turnell et al., 2005)], blocking substrate recruitment [high risk human papilloma viruses (Bellanger et al., 2005)] and altering the phosphorylation status of APC/C [human cytomegalovirus (Tran et al., 2008; Wiebusch et al., 2005)]. These inhibitory strategies focus on disrupting substrate recognition or interfering with the integrity of the complex or manipulating APC/C phosphorylation status. The orf virus strategy of replacing a core subunit of APC/C therefore differs from those reported thus far (Mo et al., 2009, 2010) and it is tempting to speculate that this might create a novel function for PACR–APC/C to serve virally. Future studies of PACR might reveal unknown functions of APC/C and knowledge gained from this could be used to manipulate APC/C for pharmaceutical interest.

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References


