

Summary: “The HPV-16 E6 and E6-AP Complex Functions as a Ubiquitin-Protein Ligase in the Ubiquitination of p53”
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The purpose of this paper was to purify and identify all of the factors that are necessary for the ubiquitination of p53. It is known that human papillomavirus (HPV) types 16 and 18 use the process of ubiquitination to target p53. In vitro experiments have also shown that HPV encodes an oncoprotein, E6, that stimulates the degradation of p53 in the presence of a 100kd protein called E6-associated protein (E6-AP).

Previous work had shown that an E6-E7 (E7 is another HPV oncoprotein) fusion protein could assist in the degradation of pRB. So, an E6-E7 fusion protein was constructed in order to assess the role of E6-AP in ubiquitination. Figure 1B shows that the fusion protein is sufficiently ubiquitinated in the presence of rabbit reticulocyte lysate (RRL) and Sf9 cells supplemented with E6-AP. Sf9 wildtype cells and what germ extracts (WGE) were not capable of ubiquitination. This suggests that E6-AP is necessary for ubiquitination of the fusion protein. – and that E6AP is only present in mammalian cell extracts.

In order to identify other factors necessary for ubiquitination, a GST fusion protein with E6-E7 was expressed in E. coli. The bacterially expressed protein fusion was not ubiquitinated by WGE, but was by RRL. The RRL was fractionated
How?? With an anion-exchange chromatography. Proteins with negative charge are retained whereas positively charged –such as Ub- elute in the flow-through (non-retained fraction). Go back to Materials from Session 2 to remember this technique.
and the fraction that contained E6-AP (fraction A) was added to WGE. This fraction was sufficient for ubiquitination with WGE. The E6-AP fraction could not ubiquitinate without WGE, so other fractions were tested.

Addition of Fraction B and the flow through from the RRL were found to be the only other fractions necessary (along with the E6-AP fraction) to cause ubiquitination of the GST fusion protein. Fraction B was then fractionated to find which of its components was contributing to the process. The fraction corresponding to what might be an E1

enzyme was found to be active. A bacterially expressed E1 was shown in Figure 3 to be capable of taking the place of fraction B. This suggests that fraction B did contain the E1 enzyme.

Additionally, a purified form of E6-AP was able to substitute for fraction A. This suggests that E6-AP is the only factor in that fraction which is required for ubiquitination. The flow through fraction was then analyzed. It is known that the flow through contains ubiquitin, but commercial ubiquitin was not able to substitute for the flow through fraction. Therefore, the flow through was further fractionated to find what other components were important. Two components, a proposed E2 enzyme and ubiquitin, were found to be active components of the ubiquitination reaction.

The E2 was found to be the mammalian homolog of UBC8. This E2 is not like other identified E2's because it does not bind to anion exchange columns. Additionally, other known E2's were not able to substitute in the ubiquitination reaction of the fusion protein.

How do they get this? They did affinity chromatography using an ubiquitin-coated column. This kind of column retains both E1 and E2, because both enzymes form a thioester with Ub. E2 needs E1 attached to Ub and is the interaction of both enzymes what allows E1 to transfer the Ub to E2 by transfer of the thioester bond between the Ub and E1 to the E2. By adding AMP + PPi they can reverse the first reaction of Ub attachment to E1 (remember this first step uses ATP and breaks it in AMP + PPi to use the energy generated in the process to form the thioester bond between the Cys and Ub). That's why this elutes the E1 but it can also elute some E2 associated to it. The addition of DTT disrupts the thioester bond between the Ub immobilized in the column and the E2 enzyme so this way is how they elute the rest of the E2 enzyme. (take another look to the steps of the Ub reaction in the introduction for Session 1 so that you can better understand this).

Once all of the components (an E1, E2, E6-AP, and ubiquitin) had been characterized and identified as capable of ubiquitinating the fusion protein, their activity was tested on a GST fusion with p53. Figure 5 showed that all of the previously identified components were necessary. If any of the components were missing, p53 was not ubiquitinated.

It also shows that E6 protein is necessary for E6AP being able to ubiquitinate p53. That's because E6AP and p53 do not interact unless E6 puts them in contact by interacting with both of them.

E6-AP was then hypothesized to be the E3 enzyme in the complex. In Figure 7, Sf9 cells with E6-AP were found to ubiquitinate cellular proteins.

From which organism are these cells?? mammalian (rabbit reticulocytes, RRL). This means that E6AP has its own cellular substrates, which are in fact different proteins and not p53.

This did not occur with non-infected or wildtype infected Sf9 cells.

All of these results allowed the authors to conclude that E6-AP was an E3 ligase that acted with the identified E1 and E2 components (and E6) to ubiquitinate p53.

It also shows that E6AP is used by HPV to ubiquitinate a non-natural substrate for this E3 ligase, the protein p53. E6 HPV acts as an ancillary protein, placing E6AP in proximity to p53 and, only then, E6AP –dependent ubiquitination of p53 can occur.