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Expression of full length Jabpol and recombinant PR in MOS human cell lines

Two DNA segments of the human jack in the box virus were cloned into a pCI vector (Promega). The first of these DNA segments coded for the complete JabPol polyprotein and the second coded for only the Jib protease (PR) protein. Both the full length JabPol and the PR coding sequences were cloned into the pCI¹ vector such that they were under the control of a cytomegalovirus (CMV) promoter. A methionine start codon was placed at the start of the region coding for the PR protein enabling translation of the protein. This is necessary because the first amino acid in the PR protein is a proline, an amino acid that cannot mediate the start of translation. The constructed plasmids were termed pCI-JabPol and pCI-PR respectively. In order to facilitate the subsequent *in vitro* experiments testing the effects of PR context on the cleavage of JabPol, a full-length JabPol variant had to be expressed that lacked the intrinsic ability for self-cleavage, which is mediated by the PR unit. The Invitrogen Genetailor site-directed mutagenesis kit was used to mutate the active site of PR to an inactive form in the pCI-JabPol plasmid. The resulting PR⁻ mutant plasmid was termed pCI-JabPolPR⁻. This site-directed mutagenesis protocol was also used to create the plasmids with a mutation of the first amino acid in the PR protein from a proline to an alanine.

Both the wild type and pro→ala mutated variants of the pCI-JabPol, pCI-PR, and pCI-JabPolPR⁻ plasmids were transfected into MOS human cells using the lipotransfection method described in previous papers². Briefly, MOS human cells were incubated overnight with lipofectamine and appropriate plasmid DNA. Post-transfection

cells were allowed to express the appropriate protein off of the constitutively active CMV promoter.

Purification of JabPol, JabPolPr- and recombinant PR proteins

In order to purify the recombinant proteins, cells were lysed in an appropriate lysis buffer and cell debris was pelleted via centrifugation. The desired proteins were purified via co-immunoprecipitation that utilized polyclonal antibodies directed against the desired protein. Once sufficient wash had insured sufficient purity of the protein, the proteins were disassociated from the antibodies bound to beads, beads were centrifuged, and the soluble phase containing the desired protein was collected. The concentration of purified protein was determined via standard Bradford Assay Protocol.

In vitro assay for the effect of PR context on cleavage

Time course *in vitro* cleavage reactions were carried out to test both the effect of PR context and the pro→ala mutation on the cleavage of the JabPol polyprotein. All reactions were incubated in conditions favorable for the cleavage of the polprotein into active products. The cleavage reactions were allowed to proceed for a set amount of time and the reactions were stopped at regular time intervals. The reactions were stopped by mixing the reaction with a denaturing SDS polyacrylamide gel loading dye solution. The following incubations were carried out to test the factors effecting cleavage order: wild-type full length JabPol, full length JabPol PR- alone (negative control), and full length JabPol PR- mutant incubated with recombinant mature PR. All of these incubations were repeated with the pro→ala mutant protein products. Once the time course experiment was run to completion, all of the samples were analyzed using SDS- polyacrylamide gel electrophoresis. Gels were stained for proteins using coomassie-blue protein stain. The

variation in fragment size as a function of PR context, time, and mutations was used to deduce the effect of context on the selection of processing sites and order of cleavage.

Special Note:

This protocol presents a special problem in the purification of the polyprotein. The polyprotein is expressed in human MOS cells; theoretically the polyprotein could undergo cleavage during either the expression steps or purification steps. In order to avoid this, one could clone the DNA coding the desired protein into an expression plasmid containing a T7 promoter. These plasmids can then be used to carry out *in vitro* transcription/ translation reactions³ in line with the cleavage experiments.

Citations:

¹ Brondyk, B. pCI and pSI Mammalian Expression Vectors. (1994) Promega Notes 49, 7.

² Gitlin, L., Karelsky, S., and Andino, R., Short interfering RNA confers intracellular antiviral immunity in human cells. (2002) Nature 418: 430-434.

³ Stemmann, O., Zou, H. and Kirschner, M. (2002) Promega Notes **80**, 2-4.