The Central Hydrophobic Domain of the Bovine Papillomavirus E5 Transforming Protein Can Be Functionally Replaced by Many Hydrophobic Amino Acid Sequences Containing a Glutamine

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The 44-amino-acid E5 transforming protein of bovine papillomavirus can induce growth transformation of cultured rodent fibroblast cell lines. Previous studies revealed that efficient transformation of mouse C127 cells by the E5 protein required a central core of hydrophobic amino acids and several specific carboxy-terminal amino acids. Although a randomly derived sequence of hydrophobic amino acids could functionally replace the wild-type hydrophobic core, most such sequences could not. We show here that the conserved glutamine at position 17 in the hydrophobic domain is also important for transformation and that insertion of the glutamine can rescue the transforming activity of many but not all otherwise defective mutants containing random hydrophobic sequences. However, a class of mutants was identified that transform efficiently even in the absence of glutamine, demonstrating that the presence of this amino acid is not absolutely required for efficient transformation. E5 proteins containing the glutamine appear to display increased homodimer formation compared with mutant proteins lacking the glutamine, but this amino acid has no apparent effect on protein stability.

The fibropapillomavirus E5 proteins are very short, membrane-associated proteins capable of causing acute and stable growth transformation of cultured fibroblasts. The bovine papillomavirus (BPV) E5 transforming protein is a 44-amino-acid protein that comprises an extremely hydrophobic amino-terminal two-thirds and a short hydrophilic carboxy-terminal tail, and it is localized primarily in the Golgi apparatus and other intracellular membranes of transformed cells (3, 4, 21). The carboxy-terminal tail of the protein appears to extend into the Golgi lumen (4), and the hydrophobic domain presumably mediates membrane association. In transformed cells, the majority of the BPV E5 protein exists as a homodimer that evidently consists of two monomers joined by interchain disulfide bonds (12, 21). In addition, the E5 protein associates with the 16-kDa subunit of the vacuolar H+ -ATPase (7, 8).

Extensive mutational and physiological analysis has been performed on the BPV E5 protein (6). Only eight specific amino acids have been identified as being required for efficient E5-mediated transformation of mouse C127 fibroblasts (12). Seven of these required amino acids are in the hydrophilic carboxy terminus and include two cysteine residues that appear to form disulfide bonds. Because the great majority of required amino acids are in the carboxy-terminal region, because these amino acids are highly conserved among E5 proteins of related fibropapillomaviruses, and because a microinjected carboxy-terminal E5 peptide can stimulate cellular DNA synthesis, it appears that this domain constitutes the active site of the protein (10, 12). The BPV and deer papillomavirus E5 proteins activate the cellular platelet-derived growth factor (PDGF) β receptor in transformed cells, and there is a short region of striking amino acid sequence similarity between the E5 active site and a portion of PDGF (14, 18). On the basis of these results, we have proposed that the E5 protein induces fibroblast transformation by activating the PDGF receptor and that activation may be mediated by direct binding of the E5 active site to the receptor (18). The E5 gene can also cooperate with cotransfected genes encoding cell surface receptors to efficiently transform NIH 3T3 cells, and the metabolism of the foreign receptors encoded by these genes appears to be altered in the transformed cells (16).

The very hydrophobic central domain of the BPV E5 protein is also required for efficient stable transformation and has been subjected to detailed mutational analysis. Initial studies of mutants containing amino acid substitutions in this region indicated that the overall hydrophobicity of this segment, rather than its precise amino acid sequence, was its major contribution to transformation (12). This conclusion was supported by the identification of a randomly derived sequence of hydrophobic amino acids that could functionally substitute for the wild-type hydrophobic core (13). Similarly, a short sequence of hydrophobic amino acids markedly stimulated the ability of a microinjected carboxy-terminal E5 peptide to induce cellular DNA synthesis (19). However, the great majority of random hydrophobic sequences tested did not support focus formation, suggesting that this portion of the protein contained some feature in addition to hydrophobicity that was crucial for transformation. Mutational analysis also suggested that the glutamine at position 17 in the hydrophobic core of the E5 protein played a role in transformation, although it could be replaced by histidine (12). Moreover, the other fibropapillomavirus E5 proteins also contain a glutamine at the homologous position (11, 17).

In this study, we have continued the genetic analysis of the hydrophobic domain of the BPV E5 protein, with special emphasis on the glutamine. We report that this amino acid plays an important but not absolutely essential role in transformation by the E5 protein and that in the presence of the glutamine, most random hydrophobic sequences are compatible with E5-mediated transformation. Further analysis of these mutants in collaboration with Schlegel and colleagues revealed that the glutamine is required for the
binding of the E5 protein to the 16-kDa component of vacuolar H\(^+\)-ATPase (9).

**MATERIALS AND METHODS**

**Plasmids and mutants.** Mutant E5 17L, containing a glutamine-to-leucine substitution at position 17 in the BPV E5 protein, was generated by oligonucleotide-directed mutagenesis by using a specific mutagenic primer and M13-based template MR7 as described before (5). After the mutation was cloned into pBPV142-6 (full-length BPV DNA [20]), the mutation was confirmed by sequencing. The marker rescue plasmid 17Lmr was constructed by replacing the small BstXI-SalI fragment of E5 17L with the corresponding wild-type fragment from pBPV142-6 (20). This manipulation is predicted to restore transformation if the mutation responsible for the defect is located in the E5 gene or in the 3' untranslated region. Mutants E5 17G (containing a glutamine-to-glycine substitution at position 17) and 15T17H (containing alanine-to-threonine and glutamine-to-histidine substitutions at positions 15 and 17, respectively) have been described previously (12).

BPV mutants directing the synthesis of E5 proteins with chimeric hydrophobic domains were constructed in ESDL12-28, which is a transformation-defective mutant with a deletion of codons 12 to 28 within the E5 gene in the 69% subgenomic transforming fragment of BPV DNA (13). Pairs of double-stranded deoxyribonucleotides, corresponding to the 5' and 3' halves of the central portion of the E5 gene, were ligated via their internal cohesive overhanging ends (after the appropriate termini were phosphorylated). Each ligation product was then inserted into the unique SpeI site at the position of the deletion in ESDL1-28, and clones containing inserted oligonucleotides were identified by colony hybridization. The correct orientation and sequence of the inserts were confirmed in each case by sequencing. For each hydrophobic sequence, versions with and without the glutamate at position 17 were constructed. Mutants HR15 and HR16 were previously designated S14-28#15 and S14-28#16, respectively (13).

Plasmids pPava-1 and -2, which contain the simian virus 40 (SV40) late region and origin of replication and the E2, E4, and E5 open reading frames of BPV have been described previously, as has PavaE5d29, which contains an E5 frameshift mutation deleting BPV nucleotides 3888 to 3916 (22, 23). Recombinant viruses directing the expression of E5 proteins harboring chimeric hydrophobic domains were constructed by substituting the small BstEII-BglII fragment of pPava-2 with the corresponding BstEII-BamHI fragment of the original mutant. Correct cloning products were confirmed by restriction digestion and DNA sequencing.

**DNA transfer and cell culture.** C127 and CMT4 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum and antibiotics. To assay the mutants for focus-forming activity, 100 ng of the full-length BPV plasmids or 500 ng of the plasmids containing the 69% subgenomic transforming fragment was digested with BamHI or with BamHI and HindIII, respectively, to separate the viral genome from the vector and transfected into C127 cells as described before (5). Foci were counted 2 to 3 weeks after transfection.

**DNA synthesis assays.** C127 cells approaching confluence in 24-well plates were infected in duplicate in DME containing 0% fetal calf serum with equivalent amounts of BPV-SV40 recombinant virus. The relative titers of the different virus stocks were determined by transactivation as described before (22). After the cells had been maintained for 48 h in serum-free medium, they were incubated for 2 h with 1.5 μCi of \(^{3}H\)thymidine per ml, and DNA synthesis was determined by measuring trichloroacetic acid-precipitable, hot perchloric acid-soluble label as described before (23).

**Immunoprecipitation of E5 protein.** CMT4 cells were infected at equal multiplicity with different recombinant Pava stocks. After 48 h, the cells were metabolically labeled for 5 h with 0.5 μCi of \(^{35}S\)methionine and \(^{35}S\)cysteine (Trans\(^{35}\)Slabel; ICN) in 1 ml of serum- and methionine-free medium. Total-cell extracts were prepared in RIPA buffer as described previously, and extracts containing equivalent amounts of incorporated radioactivity were precipitated by a modified double-immunoprecipitation procedure and a rabbit polyclonal antiserum raised against the carboxyl-terminal 16 amino acids of the BPV E5 protein (23). In some experiments, 10 mM iodoacetate was included in all steps subsequent to metabolic labeling. Immune complexes were suspended in sample buffer in the presence of 100 mM dithiothreitol (DTT) (except for the experiment shown in Fig. 4B, in which DTT was not added, solubilized by boiling, and subjected to electrophoresis on sodium dodecyl sulfate–15% polyacrylamide gels. After electrophoresis, the gels were fixed, treated with Enlightening (Du Pont NEN Research Products, Boston, Mass.), dried, and exposed to film for 3 to 14 days at ～70°C.

**RESULTS**

The experiments summarized in the introduction indicated that the overall hydrophobic nature of the middle third of the E5 protein, rather than its precise amino acid sequence, was its primary contribution to transforming activity. However, eight of the nine original random hydrophobic replacement (HR) mutants were defective for focus formation. To determine whether these HR mutants displayed a global transformation defect, we tested their ability to induce cellular DNA synthesis in quiescent cells by expressing them from a BPV-SV40 recombinant virus that induces acute, E5-mediated transformation of C127 cells. Most of the HR mutants were defective for stimulation of DNA synthesis, whereas mutant HR15 (13), which efficiently induced stable transformation, also efficiently induced cellular DNA synthesis (data not shown; see Table 2). Thus, as was the case for missense mutations throughout the E5 protein, the HR mutations affected stable transformation and induction of DNA synthesis coordinately.

To determine whether the HR mutant proteins are synthesized and stable, the E5 protein was examined in CMT4 cells infected with recombinant BPV-SV40 viruses directing the synthesis of wild-type and mutant proteins. All the E5 proteins tested were expressed at comparable levels in our steady-state labeling assay, indicating that there were no significant differences in stability (Fig. 1). Many of the random HR mutant E5 proteins display aberrant electrophoretic mobility relative to the wild-type E5 protein, as we described previously for HR15. The ATPase subunit that binds the E5 protein was not detected in any of the experiments reported here because we carried out immunoprecipitation with an antiserum recognizing the carboxy terminus of the E5 protein. Reliable coprecipitation of the ATPase subunit is only obtained by using antibodies that recognize epitopes inserted into the amino terminus of the E5 protein (7).

Previous analysis of the missense mutants indicated that
the glutamine at position 17 may also be important for transformation, although the transformation competence of hydrophobic replacement mutant HR15 showed that it is not absolutely required. To confirm that there is a true requirement for a hydrophilic residue at this position in an otherwise wild-type protein, the glutamine was replaced with a leucine to generate mutant 17L. C127 cell focus formation assays were used to compare the transformation efficiency of wild-type viral DNA with that of mutants containing histidine (15T17H), glycine (17G), or leucine (17L) in place of glutamine. As shown in Table 1, E5 proteins harboring the hydrophilic amino acid glutamine or histidine at position 17 efficiently induce C127 cell focus formation. In contrast, the neutral and the hydrophobic substitution mutations markedly inhibit stable transformation. To formally exclude the possibility that the defect associated with the glutamine-to-leucine mutation was due to additional undetected mutations elsewhere in the BPV genome, a marker rescue experiment was performed by replacing the E5 gene of the defective mutant with the wild-type gene to generate construct 17Lmr. As shown in Table 1, full transformation activity was restored by this substitution, indicating that the transformation defect associated with the leucine at position 17 is due to the constructed mutation. As was the case for the defective HR mutants and mutants with carboxyl-terminal missense mutations, 17L was also defective for acute stimulation of DNA synthesis yet synthesized a stable E5 protein (Fig. 1 and Table 2). Therefore, efficient transformation seems to require a hydrophilic amino acid in this portion of the molecule.

The experiments described above indicate that efficient E5 transformation normally requires both a hydrophobic central portion and a hydrophilic amino acid at position 17. This suggested that the transformation defect displayed by most of the HR mutants may be due to the absence of the glutamine. To determine the effect of inserting a glutamine into the random HR mutants, we constructed a series of mutant E5 genes with and without a glutamine at its normal position relative to the carboxyl-terminal active site. In addition to inserting the glutamine into a number of the original HR mutants, we also examined the activity of chimeric hydrophobic domains comprising novel combinations of the amino- and carboxyl-terminal segments of the hydrophobic domains present in several of the original mutants. Figure 2 shows the predicted amino acid sequence of the hydrophobic domain of each mutant E5 protein lacking the glutamine; the amino acid shown in boldface type was replaced with glutamine in the other member of each pair. Mutants encoding each of these proteins were assayed for focus-forming activity in C127 cells after viral DNA was excised from the plasmid vector. Multiple, independently derived isolates of most of these mutants were constructed and tested. As shown in Fig. 2, focus-forming activity of most of the hydrophobic replacement mutants was very low if the glutamine was absent and was stimulated by the insertion of the glutamine. The transformation activity of the original transformation-competent HR mutant, HR15, was high in the absence of the glutamine and was modestly stimulated by its presence, and several other mutants containing the same carboxyl-terminal hydrophobic segment as HR15 displayed a similar pattern of activity. However, a few HR mutants containing the carboxyl-terminal hydrophobic segment derived from HR16 are severely defective for transformation in the presence and absence of the glutamine. Overall, of the 16 mutant pairs tested, 13 were able to induce

<table>
<thead>
<tr>
<th>E5 backbone</th>
<th>With the glutamine</th>
<th>Without the glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>4^b</td>
</tr>
<tr>
<td>HR15/25</td>
<td>116</td>
<td>16</td>
</tr>
<tr>
<td>HR15</td>
<td>91</td>
<td>74</td>
</tr>
<tr>
<td>HR16</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>No E5</td>
<td>—</td>
<td>7^c</td>
</tr>
</tbody>
</table>

^a DNA synthesis was measured as incorporation of [3H]thymidine into DNA following infection with BPV-SV40 recombinant viruses and is expressed as a percentage of the incorporation stimulated by infection with the wild-type virus.

^b Mutant 17L.

^c Frameshift mutant E5d29.

### TABLE 2. Stimulation of cellular DNA synthesis by BPV E5 genes in the presence and absence of glutamine 17.

<table>
<thead>
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<tr>
<td>HR15</td>
<td>91</td>
<td>74</td>
</tr>
<tr>
<td>HR16</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>No E5</td>
<td>—</td>
<td>7^c</td>
</tr>
</tbody>
</table>

^a DNA synthesis was measured as incorporation of [3H]thymidine into DNA following infection with BPV-SV40 recombinant viruses and is expressed as a percentage of the incorporation stimulated by infection with the wild-type virus.

^b Mutant 17L.

^c Frameshift mutant E5d29.
MHHLMFLLFLGLV-VAAQQLLLVLLLLFLYVWDHPEC5CTGLPF

FIG. 2. Structure and transforming activity of hydrophobic replacement mutants with chimeric hydrophobic domains. The first column gives the name of the various E5 genes tested; the designation before the slash indicates the origin of the amino-terminal half of the hydrophobic domain, and the designation after the slash indicates the origin of the carboxy-terminal half. Note that wt/wt is wild type, 15/15 is HR15, and 16/16 is HR16. The second column shows the predicted amino acid sequence of the central hydrophobic domain of each of the proteins without the glutamine. The amino acid shown in boldface type is replaced by glutamine in the other member of each pair. The last two columns show the relative focus-forming activity in C127 cells of each protein with and without the glutamine as indicated. Symbols: +++, >50% of wild-type activity; ++, 20 to 50% of wild-type activity; +, 5 to 19% of wild-type activity; −, <5% of wild-type activity. The sequence of the wild-type BPV E5 protein is shown at the bottom of the figure, with the specific amino acids required for transformation shown in boldface type (12). The dash indicates the position of an additional amino acid inserted in some of the mutants.

<table>
<thead>
<tr>
<th>E5 gene</th>
<th>Hydrophobic domain</th>
<th>Transformation</th>
<th>( -\text{gln} +\text{gln} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wt</td>
<td>AAMLLLVIPLLFF</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>wt/15</td>
<td>AAMLLLVIPLLFF</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>wt/25</td>
<td>AAMLLLVIPLLFF</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>wt/16</td>
<td>AAMLLLVIPLLFF</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>15/wt</td>
<td>VIWLVGLVFLPLLFF</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>15/15</td>
<td>VIWLVGLVFLPLLFF</td>
<td>-</td>
<td>+++</td>
</tr>
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<td>15/16</td>
<td>VIWLVGLVFLPLLFF</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>16/wt</td>
<td>VIWLVGLVFLPLLFF</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>16/16</td>
<td>VIWLVGLVFLPLLFF</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>25/15</td>
<td>VIIILLVVFILLLV</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>25/25</td>
<td>VIIILLVVFILLLV</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>25/16</td>
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<td>-</td>
<td>++</td>
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<tr>
<td>16/25</td>
<td>VIIILLVVFILLLV</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

It is also possible to consider the transforming activity of individual amino- and carboxy-terminal segments of the hydrophobic domain. Each of the amino- and carboxy-terminal segments tested can support transformation if combined with an appropriate partner. However, the various hydrophobic segments differ in their ability to support transformation when combined with a variety of different hydrophobic segments. For example, the carboxy-terminal hydrophobic segment of HR16 allows transformation when it is combined with the amino-terminal hydrophobic segment of HR15 containing glutamine but not when it is combined with any other amino-terminal segment. By this measure, HR15 contains the strongest amino-terminal segment. Similarly, the carboxy-terminal segment of HR16 appears to be weaker than several carboxy-terminal segments that are rescued by diverse amino-terminal segments. However, some of the other segments cannot be unambiguously classified as strong or weak. This is illustrated by the carboxy-terminal segment of HR15. This is the only carboxy-terminal segment that can tolerate a partner without a glutamine, suggesting that it has strong transforming activity. On the other hand, this segment transforms cells poorly in combination with the amino-terminal segment of the wild type, whereas some other carboxy-terminal segments cooperate well with the wild-type segment. Thus, the ability of a hydrophobic segment to support transformation is dependent on the identity of its neighboring sequence, and the individual segments cannot be placed in a clear hierarchy.

We also tested the effects of the hydrophobic replacement mutations on acute transformation by the E5 protein. A representative pair of mutants from each of the three classes defined in terms of response to the glutamine was subcloned into the BPV-SV40 recombinant viruses and tested in an assay for acute stimulation of DNA synthesis (Table 2). The behavior of each class of mutant pair in this acute transformation assay is comparable to its behavior in the focus-forming assay; HR15 was active even in the absence of the glutamine, HR16 was defective even in its presence, and HR15/25 was defective in the absence of the glutamine and active in its presence. Thus, although each mutant class displays a different pattern of transforming activity, in no case did the presence or absence of the glutamine dissociate stable and acute transforming activity.

Representative mutants of each class were also analyzed to test the possibility that the glutamine may influence transformation efficiency by affecting the amounts of the mutant E5 proteins. As shown in Fig. 3A, the wild type and all mutant E5 proteins tested appear to be expressed at similar levels. Thus, the increased transformation activity of the mutants containing the glutamine is not due to an overall increase in the amount of the E5 proteins containing this amino acid. Similarly, the transformation-defective HR16 E5 protein containing the glutamine is present in amounts comparable to those of the other E5 proteins. Although this mutant appears to be somewhat less abundant in the experiment shown in Fig. 3A, this difference was not reproducible (see, for example, Fig. 3B). This indicates that the transformation defect of this mutant is not due to reduced protein levels.

When several pairs of E5 proteins with and without the glutamine were compared by immunoprecipitation and electrophoresis under nonreducing conditions, the version of the E5 protein containing the glutamine displayed a higher proportion of the dimer species than did the mutant without the glutamine. Whereas more than 50% of the wild-type E5 protein and all of the mutants containing the glutamine migrated as dimers, this ratio was significantly reduced in corresponding mutants that differed only by replacement of the glutamine with a hydrophobic amino acid (Fig. 3B). This effect was observed in multiple independent experiments and with all mutant pairs tested even though they contained different backbones of hydrophobic amino acids and the glutamine was replaced with three different amino acids (leucine for 17L, valine for HR15 and HR15/25, and isoleucine for HR16). The difference was most pronounced for the HR16 mutant pair, in which the monomer form is the predominant species in the absence of glutamine, whereas virtually no monomer is detectable in its presence. This phenomenon was observed if iodoacetic acid was included prior to lysis and during subsequent steps, a treatment that prevents the formation of new disulfide bonds but does not affect existing disulfide bonds, suggesting that these relative proportions of the monomer and dimer forms existed in the intact cell. Addition of DTT prior to gel electrophoresis completely converted the E5 protein to the monomeric form.
amino acid substitutions in the hydrophobic portion indicated that there were few, if any, specific sequence constraints in this portion of the molecule (12). Consistent with this conclusion, a random sequence of hydrophobic amino acids could functionally substitute for the wild-type sequence (13). On the other hand, in most cases replacement of this region with random hydrophobic amino acid sequences severely inhibited stable transformation (13). The experiments reported here resolve this apparent discrepancy by focusing on the glutamine at position 17, the sole hydrophilic amino acid in this region of the E5 protein.

Our results show that this glutamine is also important for transformation; even though it is imbedded in a hydrophobic amino acid sequence, replacing it with leucine essentially abolished focus-forming and acute transforming activity. The importance of the glutamine is further emphasized by its ability to rescue the transforming activity of a large fraction of otherwise defective mutants with random hydrophobic sequences and to stimulate transformation by some mutants with minimal transformation defects. For all mutants tested, there is an absolute correlation between acute and stable transforming activity in C127 cells, lending further support to our earlier proposal that these biological activities are mediated by a common biochemical mechanism (23). Our results indicate that many random hydrophobic amino acids can support transformation by the E5 protein so long as the glutamine or a related amino acid is also present. This confirms our previous suggestion that the wild-type sequence of hydrophobic amino acids confers little specificity that cannot be supplied by many randomly derived sequences with a similar composition (13).

The requirement for a hydrophilic amino acid at position 17 is not absolute. Some mutants with related hydrophobic domains transform cells well without the glutamine being present (although the activity of mutants with these domains appears to be enhanced by the inclusion of the glutamine); and a few mutants (also with related hydrophobic domains) are transformation defective even if a glutamine is inserted. Moreover, the activity associated with a particular amino- or carboxyl-terminal segment of the hydrophobic domain is markedly affected by the sequence of the hydrophobic segment with which it is combined. Thus, not all hydrophobic sequences are functionally equivalent. Inspection of the amino acid sequences of the hydrophobic domains tested here revealed a correlation between the number of phenylalanine residues in this region and the response of the mutants to the insertion of the glutamine: the three mutants that do not require the glutamine contain between zero and two phenylalanines, the wild-type sequence and mutants that are rescued by insertion of glutamine have between one and four phenylalanines, and the three mutants that are not rescued contain between four and six phenylalanines. In the defective mutants, multiple bulky phenylalanines may interfere with proper membrane insertion, proper packing of the E5 polypeptide with its homodimer partner, or the ability of the E5 protein to recognize it cellular target. Systematic mutagenesis of the hydrophobic domains of the wild-type E5 protein and the mutants described here should identify the features that distinguish the majority of hydrophobic sequences from those of the mutants that cannot be rescued by glutamine or that do not require the glutamine.

Our results indicate that the glutamine does not merely affect immediately adjacent amino acids but also influences distant parts of the E5 molecule. For example, the sequence of the carboxyl-terminal segment of the central hydrophobic domain of the E5 protein determines to a large extent the

DISCUSSION

Prior analysis of the biological activities of BPV E5 mutants and peptide fragments has revealed two features of the protein that are required for efficient transformation of mouse C127 fibroblasts: several specific amino acids in the carboxyl-terminal portion of the protein and the overall hydrophobic nature of the middle portion of the molecule. On the basis of these previous studies, we have identified the required carboxyl-terminal amino acids as being the active site of the molecule and have proposed that these amino acids mediate transformation by contacting and influencing the activity of a cellular protein, possibly the PDGF receptor (12, 18). Studies of mutants containing a limited number of (Fig. 3A), confirming that the signal interpreted as a dimer is not the 16-kDa protein which associates with E5 proteins containing glutamine (7, 9).

FIG. 3. Effect of the glutamine on E5 protein. The amount of E5 protein was determined in infected CMT4 cells as described in the legend to Fig. 1. (A) Samples treated with DTT prior to gel electrophoresis; (B) samples not treated with DTT. +, chimeric E5 protein containing a glutamine; −, chimeric E5 protein not containing a glutamine. Lane M, mobility markers of the indicated molecular masses (in kilodaltons).
response of the protein to the glutamine: only one carboxyl-
terminal hydrophobic segment can support transformation in
the absence of the glutamine, and a different carboxyl-
terminal hydrophobic segment is present in all the mutants
that are not rescued by the glutamine. Thus, the effect of
the glutamine (which is located in the amino-terminal half of
the central hydrophobic domain) is dependent in large part
on the amino acid sequence of the carboxyl-terminal half of
this domain. The presence of the glutamine also appears to
facilitate homodimer formation. This effect on dimerization
may be analogous to that of activating mutations in the
transmembrane domain of the product of the neu oncogene
(1). These neu mutations, which confer transforming activity
on the protein, also introduce hydrophilic amino acids in a
very hydrophobic region and appear to facilitate dimeriza-
tion (25). The hydrophobic core containing the glutamine
may participate directly in homodimer formation, as has
been shown for other transmembrane segments (2). Alterna-
tively, the effect of the glutamine on dimerization may be
mediated by the ATPase subunit bound to the E5 proteins
containing glutamine (9), or the presence of the glutamine
can directly affect the relative orientation or proximity of
the carboxyl-terminal cysteines which mediate dimer forma-
tion. It is also possible that the presence or absence of the
glutamine differentially affects the recognition of the mono-
mer and dimer forms of the E5 protein by the E5 antisera
(which was raised to a carboxyl-terminal peptide). In any
event, the presence or absence of the glutamine appears to
directly or indirectly affect the structure or conformation of
the carboxyl-terminal active site, which presumably is not in
the same local hydrophobic environment as the glutamine.

The properties of the various E5 proteins studied here are
summarized in Fig. 4. For the wild-type sequence and all HR
mutants tested, there is an absolute correlation between the
presence of the glutamine, efficient dimerization, and (as
shown and discussed in reference 9) binding to the 16-kDa
ATPase subunit. However, the presence of the glutamine and
its associated parameters does not absolutely correlate
with efficient transformation, since HR16 containing the
glutamine is transformation defective but binds the 16-kDa
protein and dimerizes efficiently, whereas HR15 transforma-
tion cells efficiently without the glutamine but displays none
of these properties. The ability of HR15 to efficiently trans-
form cells even though it does not appear to bind the 16-kDa
protein strongly suggests that this interaction is not abso-
lutely required for E5-mediated transformation of C127 cells.
Nevertheless, it remains possible that the enhanced trans-
formation activity usually conferred by the presence of
the glutamine is a consequence of increased binding of the
ATPase subunit or that the E5-16-kDa protein interaction is
important for other activities of the E5 protein.

The extremely hydrophobic composition of the central
portion of the E5 protein makes it very likely that it plays a
role in membrane localization, association, or orientation.
Thus, it is striking that there is also a glutamine residue in a
transmembrane segment of another protein associated with
the Golgi membrane, the E1 protein of avian infectious bronchitis virus (15). Recent genetic studies indicate that
the E1 transmembrane segment directs Golgi retention and that
the glutamine residue is essential for this activity (24).
However, preliminary immunofluorescence localization
studies of a number of defective HR mutant E5 proteins has
failed to reveal any obvious differences from the wild type
(7a; unpublished data). However, it is possible that diff-
ferences could emerge upon more sophisticated analysis.

The 44-amino-acid E5 protein of BPV is proving to be an
ideal candidate for structure-function analysis of a trans-
forming protein because of its small size and its potent
effects on cells. Our increasingly detailed mutational analo-
gies of the BPV E5 protein has continued to reveal unex-
pected complexity in this short protein. The functional and
biochemical study of mutant E5 proteins promises to shed
light on a number of important cellular processes, including
cell transformation and signal transduction, intracellular
trafficking, and specification of protein structure.

ACKNOWLEDGMENTS

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FIG. 4. Summary table. In the hydrophilic amino acid column, a
+ indicates that the E5 gene tested contains a hydrophilic amino
acid at position 17. In the transformation columns, a + indicates
that the E5 gene transforms cells efficiently (at least approximately 50%
as well as the wild type) as measured by the number of foci per
microgram of transfected DNA in the stable assay or by DNA
synthesis in cells infected with the BPV-SV40 viruses in the acute
assay); a − indicates that the mutants transform cells less than 15%
as well as the wild type. Some of these results are taken from
references 12, 13, and 23. In the dimerization column, a + indicates
that the dimer species of the E5 protein is the predominant one when
analyzed under nonreducing conditions; −/+ indicates that the
monomer species is predominant; nd indicates not done.