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***In vitro* dimerization of the bovine papillomavirus E5 protein transmembrane domain**

Joanne Oates[‡], Matthew Hicks[‡], Timothy R. Dafforn[§], Daniel DiMaio^{||}, and Ann M. Dixon^{‡,*}

[‡]Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom

[§]Department of Biological Sciences, University of Birmingham, Birmingham, B15 2TT, United Kingdom

^{||}Departments of Genetics, Therapeutic Radiology and Molecular Biophysics & Biochemistry, Yale University School of Medicine, New Haven, CT, 06510, USA

Abstract

The E5 protein from bovine papillomavirus is a type II membrane protein and the product of the smallest known oncogene. E5 causes tumor formation by binding and activating the platelet derived growth factor beta receptor (PDGFβR). In order to productively interact with the receptor it is thought that E5 binds as a dimer. However, wild-type E5 and various mutants have also been shown to form trimers, tetramers and even higher order oligomers. The residues in E5 that drive and stabilize a dimeric state are also still in question. At present, two different models for the E5 dimer exist in the literature, one symmetric and one asymmetric. There is universal agreement, however, that the transmembrane (TM) domain plays a vital role in stabilizing the functional oligomer, indeed mutation of various TM domain residues can abolish E5 function. In order to better resolve the role of the E5 TM domain in function, we have undertaken the first quantitative *in vitro* characterization of the E5 TM domain in detergent micelles and liposomes. Circular and linear dichroism analyses verify that the TM domain adopts a stable α-helical structure and is able to partition efficiently across lipid bilayers. SDS-PAGE and analytical ultracentrifugation confirm for the first time that the TM domain of E5 forms a strong dimer with a standard state free energy of dissociation of 5.0 kcal mol⁻¹. We have used our new results to interpret existing models of E5 dimer formation and provide a direct link between TM helix interactions and E5 function.

Tumor viruses “transform” healthy cells into cancerous cells by encoding what are known as viral transforming proteins in their genome. The primary function of these proteins is to activate cellular growth pathways, and they do this by either mimicking cell receptors or by activating native receptors (1, 2). A well-known viral transforming protein is the E5 protein from bovine papillomavirus (BPV). E5 transforms cells by binding to the platelet-derived growth factor beta receptor (PDGFβR) (3), a receptor tyrosine kinase. E5 binding results in receptor dimerization, transphosphorylation and sustained activation (4, 5), leading to uncontrolled cell growth.

E5 is the smallest known viral transforming protein containing only 44 amino acids (Figure 1a), with a highly hydrophobic central domain which partitions into the cell membrane (6, 7). Investigations carried out *in vivo* suggest that E5 must undergo homodimerization in order to efficiently activate PDGFβR. *In vivo* data also suggest that the E5 dimer interacts

*To whom correspondence should be addressed: Tel, 0 (44) 24761 50037; Fax, 9 (44) 24765 24112, ann.dixon@warwick.ac.uk.

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uniquely with the transmembrane (TM) domain of PDGF β R (8–10), in contrast to its native ligand (PDGF) which binds in the large extra-cellular domain of the receptor. This interaction is an excellent example of TM domains as contributors to specific protein-protein interactions (11–16).

In vivo mutagenesis of E5 and PDGF β R has led to identification of several amino acids in the TM domains of both proteins that are critical for complex formation and activation (9, 17–21). These results have given rise to the current model for the E5/PDGF β R complex (Figure 1b), stabilized by hydrogen bonding and electrostatic interactions of residues in both proteins (22). Mutagenesis has also suggested a critical role for disulfide bond-forming cysteine residues at the C-terminus of E5. The transforming activity of E5 can be blocked by mutagenesis of these cysteines and then restored by addition of a heterologous dimerization domain (17, 21, 23).

Despite many careful and thorough studies of this protein, there is still a lack of consensus as to the molecular organization of E5 and the driving force for oligomer formation. Thus far, *in vivo* mutagenesis (17, 20, 21), Fourier transform infrared spectroscopy (FTIR) (22), and molecular dynamics simulation (22, 24) have been applied to the study of full-length E5. From these studies, two different models have emerged for E5 – a symmetric dimer and an asymmetric dimer. The most recent model describes a symmetric dimer (Figure 1c), where E5 TM domain residues A₁₄, Q₁₇, L₂₁, L₂₄, and F₂₈ are proposed to lie at the dimer interface (21, 22, 24). The dimer is thought to be partially stabilized by interactions within the TM domain, specifically by an interchain hydrogen bond between the membrane embedded Q₁₇ residues of two monomers, a feature consistent with findings that apolar point mutations at position 17 impair E5 dimer formation *in vivo* (23, 25). In addition, this model also suggests that the C-terminal cysteine residues play a major role in E5 dimerization via disulfide bond formation (Fig. 1b). An alternative, asymmetric model for the E5 dimer has also been proposed in the literature (20). This model describes the packing of two different “faces” of the predicted TM helix, where a face composed of L₇, L₁₈ and L₂₅ is thought to pack against a face containing V₁₃, V₁₆ and L₂₀ (Figure 1d), and the authors suggest that *functional* dimers are formed via a series of stepwise rearrangements to obtain the lowest energy and optimum conformation needed to interact with PDGF β R (20).

All of the studies described above imply a central role for the TM domain of E5 in functional dimer assembly, but there is little direct experimental evidence to support this. We have previously shown that the E5 TM domain in isolation strongly oligomerizes using the TOXCAT assay (24), a powerful assay in which TM domain interactions are assayed within a natural bilayer environment. However, this assay does not report on oligomeric state. In fact, the conclusion that the native state of the E5 protein is a dimer and not a higher order oligomer rests largely on the mobility of the native protein during SDS-PAGE under non-reducing conditions. Using this method, E5 has also been shown to form trimers, tetramers, and even higher order oligomers (20).

To better resolve the assembly of E5 and the role of the TM domain in function, we present here the first quantitative biophysical evidence that the E5 TM domain forms stable dimers that can insert across membrane bilayers. We use a number of complementary biophysical techniques to report on the secondary structure, bilayer orientation, stoichiometry, and binding energy of the TM domain. The results support existing models of E5 dimers, confirm the ability of the E5 TM domain to independently drive dimerization, and highlight the critical role of this domain in function.

EXPERIMENTAL METHODS

Peptide Sequence and Purification

A peptides corresponding to the TM domain of E5 (E5_{TM}) was synthesized by the Keck Facility at Yale University using F-moc chemistry. The sequence of the E5_{TM} peptide was: KKKFLGLVAAMQLLLLLLFLFLVYWDHK; containing residues F₉-H₃₄ from E5 as well as non-native lysine residues to aid solubility and end-caps on the C- and N- termini. The peptide was purified by reversed-phase HPLC using a linear acetonitrile gradient including 0.1% trifluoroacetic acid (TFA) on a Phenomenex C4 column. The purity of pooled fractions was confirmed by mass spectrometry before subsequent lyophilisation.

Circular Dichroism (26)

CD experiments were carried out using a Jasco J715 spectropolarimeter (Jasco UK, Great Dunmow, UK) and 1 mm path-length quartz cuvettes (Starna, Optiglass Ltd, Hainault, UK), equipped with a Peltier thermally controlled cuvette holder. Spectra were recorded between 190–260 nm with a data pitch of 0.2 nm, a bandwidth of 2 nm, a scanning speed of 100 nm min⁻¹ and a response time of 1 second. The E5_{TM} peptide was prepared in 50 mM sodium phosphate buffer (pH 7.5), containing 15 mM dodecylphosphocholine (DPC) (Avanti Polar Lipids, Alabaster AL, USA) and 100 mM NaCl, to a final peptide concentration of 114 μM. CD spectra were collected at 10°C intervals between 5°C and 95°C. Data shown were averaged from four individual spectra and were truncated at 200 nm below which absorbance was too high to give reliable data. Measurement of the buffer without peptide was subtracted to obtain the final spectra.

Liposome Preparation

Liposomes containing E5_{TM} peptide were prepared by co-dissolving lipid (1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC)) (Avanti Polar Lipids, Alabaster AL, USA), and peptide in 2,2,2-trifluoroethanol (TFE), at a ratio of 10:1 lipid to peptide. The mixture was dried under nitrogen and placed under vacuum overnight to ensure complete removal of the organic phase. The resulting lipid-peptide film was reconstituted in 50 mM sodium phosphate buffer pH 7 and subjected to 5 times freeze-thaw cycles using an ethanol/dry ice bath and a water bath set at 40°C, followed by sonication at 40°C for 5 minutes.

Linear Dichroism (LD)

LD spectra were measured at room temperature between 190–400 nm. Acquisition parameters (bandwidth, etc.) were identical to those described for CD measurements. Samples were aligned in a micro-volume Couette cell (26, 27) using applied voltages of 3V, 4V, and 5V (corresponding to rotation speeds of 3K, 4K, and 5K rpm, respectively). Data shown are an average of sixteen individual scans and are truncated at 210 nm where absorbance was too high to give reliable data. Spectra of the non-aligned sample (i.e. with no rotation) were subtracted from the aligned data and smoothed (using the binomial smoothing function in Jasco spectra analysis program, version 1.53.01 [build 1]) to obtain the final spectra. The Couette cell used was built in-house; equivalent models are commercially available (Kromatek, Great Dunmow, UK).

SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS-PAGE was carried out using NuPAGE 12% Bis-tris gels (Invitrogen, Paisley, UK). Typically, samples were prepared in 50 mM sodium phosphate buffer (pH 7.5), containing 10 mM SDS and 100 mM NaCl. Peptide concentrations were estimated from the absorbance at 280 nm using a molar extinction coefficient (ϵ_{280}) of 6990 mol⁻¹ cm⁻¹ for E5_{TM}. Peptide visualization was achieved either by staining with Coomassie-G250 or with silver nitrate.

Analytical Ultracentrifugation

Sedimentation equilibrium and sedimentation velocity measurements were carried out on a Beckman XL-I/A analytical ultracentrifuge (Beckman Coulter, Fullerton CA, USA). E5_{TM} peptide samples were prepared in 50 mM sodium phosphate buffer (pH 7.5), containing 15 mM DPC (Avanti Polar Lipids, Alabaster AL, USA), 100 mM NaCl, and 52.5% D₂O (Cambridge Isotope Laboratories, Andover MA, USA) to match the buoyant density of the detergent. When the solvent matches the buoyant density of the detergent micelles, the only contribution to the buoyant molecular weight is from the peptide, as described (28). Data were collected at 25°C using absorbance optics set to 280 nm. For sedimentation equilibrium a six-channel centerpiece was used. Measurements were taken at three peptide concentrations (29, 72, and 114 μM) and three speeds (37000, 40000 and 43000 rpm). Samples were centrifuged for time periods in excess of those required to reach equilibrium (20 hours per speed), and establishment of sedimentation equilibrium was verified using the program WinMatch to subtract successive scans until no difference was observed. The E5_{TM} peptide monomeric molecular mass and partial specific volume in 52.5% D₂O were determined to be 3685 Da (corrected for H-D exchange) and 0.8059 ml g⁻¹, respectively using the program SEDNTERP (available at <http://www.rasmb.bbri.org/>). Global fitting of the nine data sets was undertaken by nonlinear least-squares curve-fitting using Win-NONLIN (29). Assuming that the contribution of any bound detergent is negated by the addition of D₂O, the data were fit to Equation 1 below:

$$c_{total} = c_{monomer, r_0} e^{\left[\frac{\omega^2}{2RT} M(1-\bar{v}\rho)(r^2 - r_0^2) \right]} + c_{n-mer, r_0} e^{\left[\frac{\omega^2}{2RT} nM(1-\bar{v}\rho)(r^2 - r_0^2) \right]} \quad (1)$$

where c_{total} is the total absorbance at a radial position, $c_{monomer}$ is the monomer absorbance at a reference position, r_0 ; M is the monomer molecular mass; $\bar{v}$ is the monomer partial specific volume; ρ is the solvent density (calculated as 1.059 g ml⁻¹ as reported previously (30)); ω is the angular velocity (radians second⁻¹); R is the universal gas constant; T is the absolute temperature; c_{n-mer} is the n -mer absorbance at a reference position, r_0 . The value of n is chosen by the operator to represent the stoichiometry required for fitting. Goodness-of-fit was determined by examination of the spread of the residuals around the best-fit and minimization of variance. As a single global association constant could be used to describe the data the reaction was shown to be reversible on the time scale of the experiment. The molar association constant for a monomer / dimer equilibrium (k_{1-2}) was calculated according to Equation 2, assuming that the extinction coefficient of the n -mer is n times that of the monomer:

$$k_{1-2, conc} = k_{1-2, abs} \epsilon l / 2 \quad (2)$$

where $k_{1-2, abs}$ is the association constant in absorbance units; ϵ is the molar extinction coefficient and l is the path length. The apparent free energy of dissociation (ΔG_{app}) and the mole fraction standard state free energy of dissociation (ΔG_x^0 ; which accounts for detergent concentration) were calculated according to Equations 3 and 4, respectively (31):

$$\Delta G_{app} = -RT \ln K_{app} \quad (3)$$

$$\Delta G_x^0 = \Delta G_{app} - RT \ln [\text{micellarDet}]_w \quad (4)$$

where K_{app} is the apparent dissociation constant ($1/k_{1-2, conc}$) obtained from the fit and $[\text{micellarDet}]_w$ is the concentration of detergent in the micellar phase.

Sedimentation velocity data were collected at a peptide concentration of 114 μM using a double-channel centerpiece and a speed of 40000 rpm. 400 scans were recorded in each case, with 50 seconds between each scan. Fitting of the resulting profiles was achieved using SEDFIT (32) to generate a continuous sedimentation coefficient distribution, which was subsequently converted to a molecular mass distribution. The buffer density was the same as that used for the equilibrium experiment and the buffer viscosity was calculated as 1.01026 cP using SEDNTERP.

RESULTS

The E5_{TM} domain forms a thermally stable α -helix in DPC micelles

To investigate TM domain interactions of the E5 protein *in vitro*, we synthesized a peptide corresponding to the E5 TM domain (E5_{TM}, residues F₉-H₃₄). Lysine residues were added at the N- and C-termini to aid solubility of this highly hydrophobic peptide and reduce non-specific aggregation. This approach has been shown in a number of studies not to affect the properties of strongly interacting TM domain peptides (33–35). However, for more weakly interacting TM helices, there are examples in the literature in which lysine and other positively charged residues abolish dimerization, and should therefore be used with caution (36, 37).

The full-length E5 protein has been shown to be predominantly α -helical in synthetic membrane bilayers using FTIR (22). To determine the secondary structure of the E5_{TM} peptide in detergent micelles, CD spectra were acquired for peptide reconstituted into the zwitterionic detergent dodecylphosphocholine (DPC). The resulting spectra (Figure 2) show a characteristic α -helical profile, with negative absorption maxima at 208 and 222 nm, demonstrating that the E5_{TM} peptide is α -helical in DPC micelles. Fitting of CD data collected at 25°C using K2d software (38) indicates that the peptide is approximately 88% α -helical. This helical secondary structure is very stable over a large temperature range. Only a small decrease in α -helical structure was observed between 5°C-95°C and the process was completely reversible (see dotted line in Figure 2), a phenomenon that has been observed previously for other detergent-solubilized TM peptides (39).

E5_{TM} peptide inserts into lipid vesicles

FTIR was also used to demonstrate that the full-length E5 protein inserts into synthetic lipid bilayers *in vitro* (22). To investigate whether the α -helical E5_{TM} peptide retains the ability of the full-length protein to effectively partition into lipid bilayers (22), we have employed the solution-phase method of linear dichroism spectroscopy (LD) (40, 41). Recent technical advances in LD have enabled the use of small sample volumes (< 50 μL) (26, 27), facilitating its application to study of membrane proteins (41). The signal obtained in LD results from the difference in absorption of horizontally and vertically polarized light by a sample aligned parallel to one of the polarization axes (usually the horizontal direction). Therefore samples for LD must be capable of being aligned, which means that the molecules under investigation must be asymmetric in either charge or shape. Liposomes are well suited to this technique as they can be flow-aligned in a rapidly spinning Couette cell to produce elongated shapes (Figure 3a) (41, 42). If there is no interaction between peptide and liposomes, the peptide will not align and therefore no difference in the absorption of the two polarizations of light (LD signal) will be observed (Fig. 3a). If the peptide inserts as an α -helix across the bilayer (i.e. orients perpendicular to the membrane), a broad positive LD signal centered at approximately 230 nm (absorbance maximum for peptide bond) will be observed, whereas peptides lying on the surface of the membrane (i.e. oriented parallel to the bilayer) will yield a negative LD signal at this wavelength. (40, 41).

We investigated the interaction between the E5_{TM} peptide and synthetic lipid bilayers by acquiring LD spectra of the peptide in flow-aligned DMPC liposomes and, as a control, in DPC micelles. The LD spectrum of DMPC liposomes was also recorded, and no absorption in the wavelength range of interest was observed (data not shown). Figure 3b shows the resulting LD spectra collected at different rotation speeds for the E5_{TM} peptide in DMPC bilayers. In all three spectra, we observe a positive absorption peak centered at 230 nm, demonstrating that the E5_{TM} peptide is preferentially inserted across the membrane bilayer. The positive gradient across the spectra for the liposome-containing samples is due to an increase in light scattering with decreasing wavelength. The increase in peptide LD signal intensity with increasing rotation speed is due to the increase in alignment of the liposomes, demonstrating that the LD signal of the peptide is dependent on alignment as expected for a peptide inserted across the bilayer. The small positive peak at ~290 nm results from absorbance of the tryptophan sidechain, indicating that this sidechain is ordered in the peptide-membrane complex. As expected, there is negligible LD signal in the micelle sample (Fig. 3b) as micelles are too small to align.

The E5 TM domain forms stable homodimers

In order to determine the oligomeric state of the E5 TM domain, SDS-PAGE was used to analyze E5_{TM} peptide samples over a range of concentrations (3–173 μ M). As shown in Figure 4a, Coomassie blue was only able to detect the E5_{TM} peptide at concentrations above 17 μ M (Figure 4a). Above this concentration, the peptide predominantly runs as a single discrete band with a molecular weight of approximately 10 kDa. Only at the highest concentration of total peptide (173 μ M) is a second weak band visible at approximately 5 kDa.

This very weak band becomes more easily visible upon silver staining (Figure 4b), a method that is well-known to be 100–1000 times more sensitive than staining with Coomassie blue. It is difficult to isolate the exact source of this enhanced sensitivity, as the binding mechanisms involved in silver staining still remain unclear (43). However, studies thus far suggest that silver ions coordinate to the protein via high affinity binding sites known to be very active in metal binding, such as amino, imidazole, and sulphhydryl groups on amino acid sidechains (44). In contrast, Coomassie blue dye binds to basic and aromatic amino acids via non-covalent interactions, the affinities of which depend heavily on the identity of the amino acid and the protocol used (45). Perhaps most importantly in the analysis of membrane proteins, Coomassie binding is sensitive to interference from detergents (46).

The positions of the E5_{TM} peptide bands at 5 and 10 kDa on the SDS-PAGE gel do not correspond exactly with the monomer and dimer molecular weights predicted from the sequence (3.66 and 7.32 kDa, respectively). Similar aberrant behavior on SDS-PAGE gels has been documented for other membrane proteins, and is thought to stem from conformational effects (47), making data interpretation difficult. Nevertheless, the two bands in the SDS-PAGE gel of the E5_{TM} peptide were thought to correspond to monomer (at 5 kDa) and dimer (at 10 kDa). The fact that we see two distinct bands in the gel also suggests that exchange between the two states is slow relative to the timescale of the experiment, and the association is reversible (13).

The SDS-PAGE results suggest that a monomer/dimer equilibrium exists for the E5 TM domain, but that this equilibrium is heavily weighted towards formation of dimers. The mechanisms underlying formation of SDS-stable dimers are not well-understood, but we do know that the chemical properties of detergents play a key role in the lateral association of TM helices (48). In many cases, SDS is unable to completely denature these transmembrane peptides, indeed experiments have shown that SDS is able to induce formation of helical secondary structure during membrane protein refolding (49–51). Additionally, positively

charged residues in the peptide (K, R, H) can interact with the negatively-charged head group of SDS and, depending on the position of the residue in the sequence, these interactions could potentially have either a stabilizing or destabilizing effect (37).

In order to more quantitatively determine the oligomeric state of the TM domain and derive thermodynamic parameters for the interaction, the E5_{TM} peptide was analyzed by analytical ultracentrifugation (52). AUC is a technique that is rapidly gaining recognition as a powerful tool in the study of membrane protein interactions. Both sedimentation equilibrium and sedimentation velocity experiments were employed in this study, as they yield complementary information about the size and shape of proteins. Sedimentation equilibrium analyses were carried out at three speeds and three peptide concentrations in DPC detergent micelles, and the concentration versus radial distance profiles for each set of conditions are shown in Figure 5. The data were initially fit to a monomer model, followed by fitting to models of increasing complexity as described (53, 54). The model of best fit is identified when the square root of variance (SRV) of the fit is minimized and the residuals of the fit are distributed randomly about zero. The statistics from each fit are summarized in Table 1, showing that a minimum value for the SRV was obtained when the data were fit to either a monomer / dimer model or a monomer / dimer / tetramer model. Both of these models also resulted in the most random fit residuals. The monomer / dimer model was further supported by fitting of the data to a single species model, which yielded a molecular weight close to that of dimer. In all models tested, inclusion of a non-ideality factor was required in the analysis to obtain a good fit to the data. Inclusion of a non-ideality parameter was justified in this case as a decrease in the weight-average molecular weight was observed with increasing concentration. Non-ideal behavior of an associating system can result from charge or crowding effects and is incorporated into the model using the second virial coefficient, *B*. In the case of protein detergent complexes, non-ideality most likely results from the detergent itself (55) or from electrostatic interactions (56).

The results of the sedimentation equilibrium experiments allowed us to narrow down the possible oligomeric states of the E5 TM domain. In order to select from the two best-fit models, sedimentation velocity analyses were also conducted using identical buffer conditions. Sedimentation velocity can be difficult with peptides of low molecular weight due to the high speeds necessary to minimize the rate of back diffusion; however a speed of 40,000 rpm was sufficient to produce data with the required mass resolution. Fitting residuals and sedimentation profiles are shown in Figures 6a and 6b respectively, with the fit data corresponding to an RMSD of 9.75×10^{-3} . The resulting sedimentation coefficient profile contains a single species centered at *S* = 0.275 (Figure 6c), corresponding to a molecular mass for the E5_{TM} peptide of 7.27 kDa (which agrees to within 1% of the calculated molecular weight of the dimer, 7.32 kDa). This fit produced a frictional ratio of 2.54, suggesting a significantly elongated ellipsoidal shape for the E5 TM domain dimer, as would be expected for rod-shaped TM α -helices.

Because the sedimentation velocity analyses clearly indicated that E5_{TM} was predominantly a dimer, we used the monomer / dimer model to determine dissociation constants from the sedimentation equilibrium data. Residuals from global fitting of the data to a single monomer / dimer dissociation constant are shown in Figure 5. Analysis of the dimer fit yielded an apparent monomer / dimer dissociation constant ($K_{d, \text{app}}$) of 4 μM in 15 mM DPC and a corresponding apparent free energy of dissociation $\Delta G_{\text{app}} = 7.4 \text{ kcal mol}^{-1}$ at 25°C. To facilitate comparison of this value to other published data and take into account the detergent concentration (which can significantly effect the measured energetics of TM dimerization), ΔG_{app} was then converted to a mole fraction standard state free energy of dissociation as described in Equation 4 and thoroughly in reference (31). This conversion

resulted in a free energy of dissociation of 5.0 kcal mol⁻¹ for the E5 TM dimer (as compared to 7.0 kcal mol⁻¹ for GpA (31)).

In combination, the *in vitro* data from SDS-PAGE and AUC clearly demonstrate for the first time that the E5 TM domain forms a strong dimer in both SDS and DPC detergent micelles, providing strong evidence for the central role of the TM domain in E5 homodimer formation and function.

DISCUSSION

In vivo studies of E5 have implicated the TM domain in dimerization and function, but its precise role in this process remained unclear. (20–22, 24). We have previously reported that the TM domain of E5 strongly self-associates in natural membranes using the TOXCAT assay (24). Furthermore, we found that the self-association of the TM domain was highly dependent on a centrally located glutamine residue (Q₁₇); a property that has also been observed in the full-length protein. (25). However, TOXCAT can only provide a relative measure of affinity (relative to GpA) and does not provide a quantitative measure of either the oligomeric state or the thermodynamic properties of the process.

Here, we report the first investigation of the E5 TM domain *in vitro*. This approach has allowed us to directly determine the oligomeric state of the E5 TM and thus its contribution to formation of a functional E5 oligomer. We have observed experimentally the ability of the α -helical E5 TM domain to insert into lipid bilayers approximately parallel to the membrane normal using Couette flow linear dichroism spectroscopy. We have also observed the ability of the TM domain to form SDS-stable homodimers, properties it shares with the full length protein *in vivo* and *in vitro* (6, 22). Furthermore, analytical ultracentrifugation has provided the first thermodynamic description of E5 TM dimerization in DPC detergent micelles. We report a free energy of dissociation (ΔG_d) of 5.0 kcal mol⁻¹, which is comparable to other strongly dimerizing TM domains (i.e. GpA) (53). These data allow us to say with some confidence that the information encoded within the E5 TM domain is sufficient to drive insertion and dimerization of the protein within biological membranes, a conclusion implied in previous studies (20, 21) but not demonstrated experimentally until now. It is likely that C-terminal disulfide bonds in E5 contribute to stability of the dimer *in vivo*, but probably do not define the structure.

In focusing our attention specifically on the TM domain, we have begun to unravel the mechanism of E5 function by providing a quantitative description of the TM driven dimerization process. The ability of the TM domain to form strong dimers ($K_{d,app} = 4 \mu M$) has significant implications for future structural studies of E5, which would be greatly simplified by removal of the cysteine residues. Understanding the precise requirements for E5 dimerization is essential in unraveling the molecular details that govern the interaction of this protein with PDGF β R and ultimately its ability to transform cells. Furthermore, study of the critical role of TM domain interactions in this system may also shed new light on the fundamental principles which govern TM-mediated interactions between membrane proteins.

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ABBREVIATIONS

AUC	analytical ultracentrifugation
BPV	bovine papillomavirus
CD	circular dichroism
DOF	degrees of freedom
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPC	dodecylphosphocholine
FTIR	fourier transform infrared spectroscopy
LD	linear dichroism
MRE	mean residue ellipticity
PDGF	platelet derived growth factor
RMSD	root-mean-square deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRS	sum of residuals squared
SRV	square root of variance
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TM	transmembrane

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Figure 1.

(a) Sequence of full-length BPV E5 protein. The predicted transmembrane domain is highlighted (shaded box). (b) The current model of the E5/PGF β receptor complex, showing the interaction of a disulfide-bonded E5 homodimer with the transmembrane domain of the receptor. Mutagenesis suggests that this complex is stabilized by electrostatic interactions between D₃₃ of E5 and K₄₉₉ of the receptor as well as hydrogen bonding between Q₁₇ of E5 and T₅₁₃ of the receptor. (c) Current model of the E5 homodimer, as shown in panel (b). The symmetrical dimer is stabilized by C-terminal disulfide bonds and interhelical hydrogen bonding of Q₁₇, and contains residues A₁₄, Q₁₇, L₂₁, L₂₄, and F₂₈ at the dimer interface. (d) The asymmetric model of the E5 dimer, stabilized by the packing of L₇, L₁₈ and L₂₅ on one monomer against V₁₃, V₁₆ and L₂₀ of another monomer.

Figure 2. Circular dichroism spectra of purified E5_{TM} synthetic peptide solubilized in buffer containing 15 mM DPC. Individual spectra (solid lines) were collected while increasing the temperature from 5°C to 95°C in 10°C increments using a Peltier thermally controlled cuvette holder. Reversibility of melting was confirmed by cycling the temperature back to 5°C and re-measuring the spectrum (dashed line). Data is reported in units of mean residue ellipticity (MRE).

Figure 3.

(a) Linear dichroism of peptides in liposomes relies on the alignment of liposomes by shear flow, with the parallel direction defined as the flow direction (top row). Peptides are too small to align by themselves and only give an LD signal when associated with the aligned liposomes (middle row). Different orientations of peptide will result in different average directions for transition moments of the peptide. For example the n to π^* transition of the peptide bond in an α -helix (around 230 nm) is oriented perpendicular to the helix axis, and will give rise to the LD signals indicated in the bottom part of the diagram. Note that the lower energy π to π^* transition of the peptide bond in an α -helix (around 210 nm) should give the opposite sign signal. (b) Linear dichroism spectra of E5_{TM} peptide in micelles and in liposomes: E5_{TM} peptide in DPC micelles rotating at 5000 rpm (dotted line), E5_{TM} peptide in liposomes rotating at 5000 rpm (dash-dot), 4000 rpm (dash) and 3000 rpm (solid line).

Figure 4. SDS-PAGE results for purified E5_{TM} and GpA_{TM} peptides run against molecular weight standards (MW). (a) Analysis of the E5_{TM} peptide was carried out over a range of concentrations (stated below each lane) and yielded primarily dimers (dimer MW=7.32 kDa), labeled *d* in the Coomassie stained gel, with only a trace of monomer (*m*) visible at the highest concentrations. (b) The presence of E5_{TM} monomers was more clearly revealed by silver staining.

Figure 5.

Sedimentation equilibrium data obtained for E5_{TM} peptide in buffer containing 15 mM DPC and 52.5% D₂O. Shown in the lower three plots are the data collected at three concentrations, ranging from 114 μ M to 29 μ M. At each concentration, data were collected at three speeds (37,000, 40,000, and 43,000 rpm). Filled circles represent experimental data and solid curves display the best fit resulting from global analysis of all nine data sets, in this case the fit of a model representing a monomer / dimer equilibrium. Above each plot are the residuals of the fitting process.

Figure 6.

Sedimentation velocity data obtained for E5_{TM} peptide in buffer containing 15 mM DPC and 52.5% D₂O. (a) Residuals of the fitting process. (b) Raw data showing the sedimentation profiles of the peptide over the time course of the experiment. (c) Sedimentation coefficient distribution profile as calculated using the SEDFIT program. Conversion of sedimentation coefficients to molecular mass yielded a peak value of 7.27 kDa, which agrees closely to the theoretical mass for the dimer (7.32 kDa).

Table 1

Non-linear least squares fitting statistics^a for sedimentation equilibrium data and resulting dissociation constants^b for E5 TM domain.

Fit	SRV ($\times 10^{-3}$)	DOF	K_d (M) dimer	K_d (M) tetramer
M	223	586	-	-
M/D	7.60	584	4.4×10^{-6}	-
M/Tr	8.58	584	-	-
M/Tet	9.72	584	-	-
M/D/Tet	7.63	584	4.1×10^{-6}	3.0×10^{-10}

^aStatistics are given for fits to monomer (M), monomer-dimer (M/D), monomer-trimer (M/Tr), monomer-tetramer (M/Tet) and monomer-dimer-tetramer (M/D/Tet) equilibria. Variance of fit, sum of residuals squared (SRS), square root of variance (SRV), and the number of degrees of freedom in the fit (DOF) are shown.

^bAlso shown are the apparent dissociation constants (K_d) as calculated from the fits for both dimer and tetramer.