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E5 oncoprotein retained in the endoplasmic reticulum/*cis* Golgi still induces PDGF receptor autophosphorylation but does not transform cells

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The E5 oncoprotein encoded by bovine papillomavirus type 1 is a homodimeric, hydrophobic polypeptide which is localized predominantly in Golgi membranes and which transforms several cell types apparently by inducing tyrosine phosphorylation of the platelet-derived growth factor receptor (PDGF-R). While the precise mechanism of receptor activation is unknown, E5 associates with several cellular proteins, including PDGF-R and the 16K V-ATPase protein, and induces the preferential phosphorylation of immature, Endo H-sensitive forms of the receptor. To evaluate whether E5 accumulation in the Golgi was requisite for receptor phosphorylation and cell transformation, we sequestered the E5 protein in the endoplasmic reticulum (ER)/*cis* Golgi by appending the ER retention KDEL sequence to its C-terminus. In transient assays and in cell lines, E5/KDEL protein and E5/KDEL* protein (a defective variant of KDEL), were stable and formed homodimers normally. E5/KDEL*, similar to wt E5, localized to the Golgi and was transformation-proficient. In contrast, E5/KDEL failed to concentrate in the Golgi and was transformation-incompetent. Despite these critical defects, however, E5/KDEL formed stable complexes with immature PDGF-R and 16K and, even more unexpectedly, induced the phosphorylation of both mature and immature PDGF-R on tyrosine residues to the same level as wt E5. These data demonstrate that E5 can bind and induce PDGF-R phosphorylation in the ER/*cis* Golgi, but that successful mitogenic signalling (and consequent cell transformation) requires the translocation of E5/receptor complexes to distal Golgi compartments.
Key words: E5 oncoprotein/endoplasmic reticulum/KDEL/PDGF receptor/transformation

Introduction

Papillomaviruses (PV's) infect a wide range of vertebrate hosts (Band *et al.*, 1990) and induce benign papillomas and fibropapillomas of squamous epithelium. These double-stranded DNA viruses are remarkably similar in their structure and genetic organization and, in humans, ~70 different types have been identified. A subset of HPV's are closely associated with the development of cervical dysplasia and carcinoma (Orth, 1987; zur Hausen and Schneider, 1987).

The bovine papillomavirus type 1 (BPV-1) genome was the first PV to be sequenced and genetically dissected to identify its transforming genes (Dvoretzky *et al.*, 1980; Lowy *et al.*, 1980; Chen *et al.*, 1982). The major *in vitro* transforming protein in BPV-1 is E5 which, at 44 amino acids in length, is the smallest known viral oncoprotein. When E5 is expressed in immortalized murine cells, they become morphologically transformed, proliferate in an anchorage-independent manner and form tumors in nude mice (Dvoretzky *et al.*, 1980; Tada *et al.*, 1989). Structurally, E5 can be divided into two domains. The 30 N-terminal amino acids are hydrophobic and are thought to exist in an α-helical conformation important for membrane anchoring and cell transformation (Burkhardt *et al.*, 1987; Schlegel and Wade-Glass, 1987; Horwitz *et al.*, 1988). E5 is a type II transmembrane (TM) protein; it is localized asymmetrically in Golgi membranes with its C-terminus being oriented toward the Golgi lumen (Burkhardt *et al.*, 1989). The C-terminal 14 amino acids are generally hydrophilic and contain two cysteine residues which are important for homodimerization and transforming activity (Burkhardt *et al.*, 1987; Schlegel and Wade-Glass, 1987; Horwitz *et al.*, 1988).

The molecular mechanism of E5-mediated transformation is not completely understood, but it appears to depend upon the phosphorylation of growth factor receptors. Functionally, E5 can co-operate with the epidermal growth factor receptor (EGF-R) and colony stimulating factor-1 receptor for transforming fibroblasts, and can induce the hyperphosphorylation/activation of EGF-R by a ligand-independent mechanism (Martin *et al.*, 1989). E5 can also physically associate with EGF-R (Cohen *et al.*, 1993a). However, in most cases, cell transformation by E5 appears to require the presence of the platelet-derived growth factor receptor (PDGF-R) (Petti *et al.*, 1991; Petti and DiMaio, 1992, 1994; Nilson and DiMaio, 1993; Goldstein *et al.*, 1994). E5 binds and induces autophosphorylation of PDGF-R specifically (Petti *et al.*, 1991; Petti and DiMaio, 1992) and forms a ternary complex with PDGF-R and the 16K subunit of the vacuolar H^+-ATPase (V-ATPase) which is mediated by the TM domains of these proteins (Goldstein *et al.*, 1992a; Cohen *et al.*, 1993a). E5 does not appear to bind to PDGF-R by mimicry of PDGF (Meyer *et al.*, 1994), as previously suggested (Petti and DiMaio, 1992). The simplest explanation for E5 transforming activity is that E5 dimers bind to PDGF-R via TM domains and effect receptor cross-linking and phosphorylation (Goldstein *et al.*, 1992a). The observation that immature forms of PDGF-R are the prominent target for E5-mediated phosphorylation is consistent with E5 being most abundant in the Golgi. In addition, the prominence of 16K in the Golgi is consistent with its apparent recruitment into these E5/PDGF-R complexes (Goldstein *et al.*, 1992a). Therefore, while it may not be the only

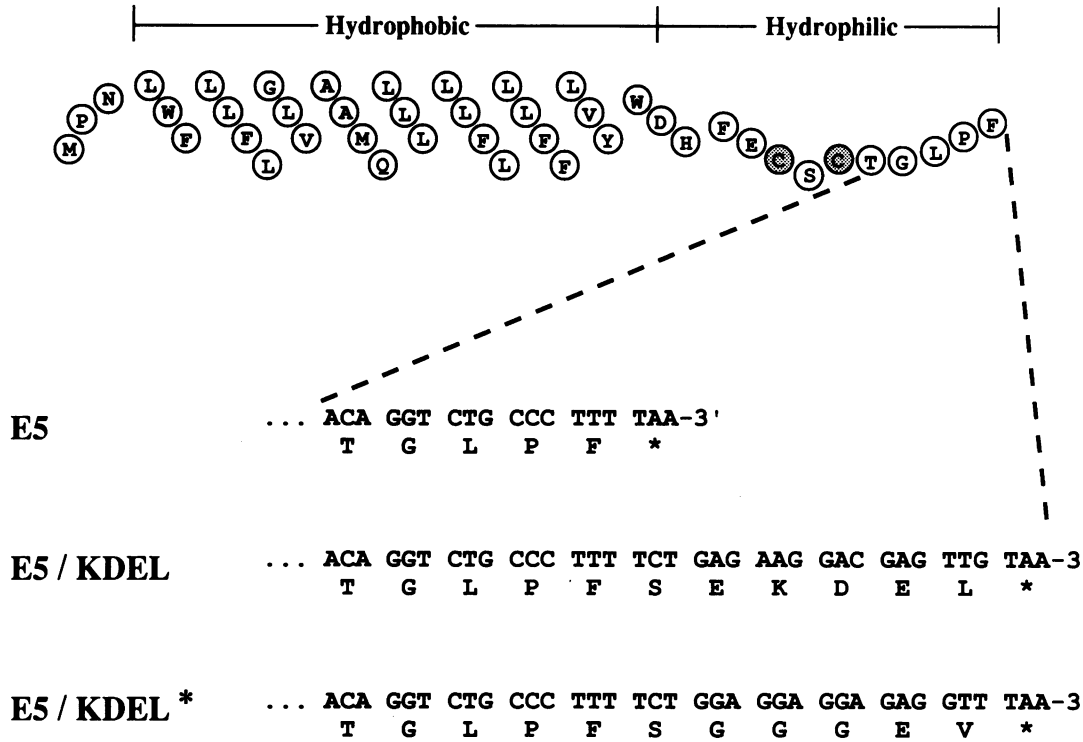

Fig. 1. Amino acid sequence of wt and mutant E5 proteins. The top panel shows the amino acid sequence of E5 which can be divided into two distinct domains: the 30 N-terminal residues which are hydrophobic and thought to be involved in membrane insertion and the C-terminal 14 amino acids which are hydrophilic and contain two cysteine residues important for homodimerization and biological activity. The bottom panel displays a comparison of C-terminal sequences of the wt E5 with those of E5/KDEL and E5/KDEL*. These constructs were cloned into a vector containing the SV40 early promoter (see Materials and methods) for expression in monkey and mouse cells.

route for E5 to induce mitogenesis, the PDGF-R signal transduction pathway appears to be a major one.

To determine if Golgi localization is required for E5-induced PDGF-R phosphorylation and cell transformation, we constructed two E5 mutants: one containing a wild-type C-terminal KDEL sequence for retaining E5 in the ER and one containing a multiply-mutated KDEL sequence with substitutions at amino acids essential for ER retention (Munro and Pelham, 1987). KDEL sequences, which are highly conserved in yeast and mammalian cells, mediate the interaction of endogenous ER proteins with a specific receptor that recycles proteins from the *cis*-Golgi back to the ER (Townsley *et al.*, 1993). We appended the KDEL motif to the E5 C-terminus for the following reasons: (i) the C-terminal location would permit ready interaction with the KDEL receptor, (ii) the E5 C-terminus tolerates the addition of amino acids without significant effects on transforming activity and (iii) another type II membrane protein, dipeptidy peptidase IV, can be sequestered efficiently in the ER by a C-terminal KDEL signal (Tang *et al.*, 1992b). Our results indicate that, while E5/KDEL can initiate PDGF-R autophosphorylation, cell transformation does not occur, suggesting that the ER/*cis*-Golgi compartment lacks critical substrate interaction(s) necessary for successful PDGF-R mitogenic signalling.

Results

Construction and expression of E5/KDEL and E5/KDEL mutants*

In order to determine whether Golgi localization was required for E5 transforming activity, we used PCR techniques to construct an E5 mutant, E5/KDEL, which was designed for retention in the ER by the addition of a C-terminal, ER retention hexapeptide, SEKDEL (Figure 1). Appending the KDEL peptide to the hydrophilic, lumen-facing E5 C-terminus permits interaction with the KDEL receptor (Burkhardt *et al.*, 1989; Singh *et al.*, 1993).

To evaluate whether the simple addition of six amino acids at the E5 C-terminus would interfere with Golgi localization and cellular transformation, a second mutant, E5/KDEL*, was constructed. E5/KDEL* contains a modified C-terminal KDEL sequence (SGGGEV) which lacks four critical amino acids necessary for ER retention and recognition by the KDEL receptor (Munro and Pelham, 1987). Both E5/KDEL and E5/KDEL* mutants contain an additional N-terminal epitope, AU1, to facilitate their immunologic detection and isolation. Utilization of this epitope for immunoprecipitation was particularly important since the C-terminal KDEL additions interfered with the immunoprecipitation of E5 with rabbit antisera directed against the E5 C-terminus (data not shown). The N-terminal AU1 epitope does not interfere with E5 biological activity or intracellular localization (Sparkowski *et al.*, 1994).

E5, E5/KDEL and E5/KDEL* were cloned into pJS55, a derivative of pSG5 (Stratagene) (Sparkowski *et al.*, 1994), which utilizes the SV40 early promoter for efficient expression in both primate COS cells and mouse fibroblasts (C127 or NIH3T3 cells).

The wild-type and KDEL E5 clones were initially evaluated for protein expression in COS cells. Forty-eight hours post-transfection, COS cells were lysed, immunoprecipitated with AU1 antibody and the immunoprecipi-

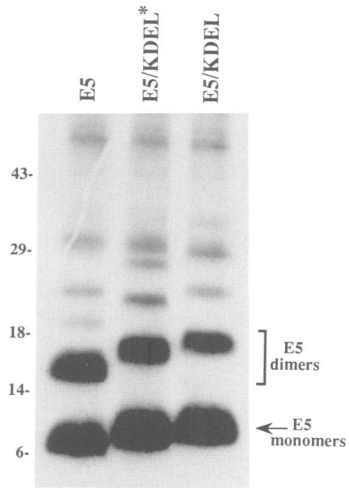

Fig. 2. E5, E5/KDEL* and E5/KDEL proteins are stable and dimerize normally. COS cells were transiently transfected with each of the E5 constructs, metabolically labelled with [^{35}S]cysteine, immunoprecipitated with AU1 monoclonal antibody and analyzed on SDS–PAGE gels without reducing agents as described in Materials and methods. Monomer and dimer forms of E5 are indicated on the right. Molecular weight markers (in kDa) are listed on the left.

tates separated on SDS–PAGE gels lacking reducing agents. As shown in Figure 2, the wild-type, E5/KDEL* and E5/KDEL proteins were all expressed at similar levels in COS cells and retained their normal capacity to form homodimers. The ability of the E5 mutants to form homodimers also suggested that they were oriented normally in membranes with their C-termini facing the Golgi lumen (Burkhardt *et al.*, 1989). The formation of E5 homodimers via disulfide bond formation between C-terminal cysteine residues is dependent upon an oxidizing environment characteristic of the ER and Golgi compartments.

E5/KDEL localizes to the ER rather than to the Golgi

Results from previous immunofluorescence and immunoelectron microscopy studies indicate that E5 resides predominantly in the Golgi (Burkhardt *et al.*, 1989). To determine if the addition of KDEL to E5 would prevent normal Golgi accumulation and target it to the ER, we used similar indirect immunofluorescence techniques. COS cells were transfected with E5 DNA constructs and evaluated by immunofluorescence as described in Materials and methods. Figure 3 demonstrates the normal Golgi localization of wt E5 (panel 4). In contrast, E5/KDEL did not accumulate in the Golgi and was observed in a reticular, cytoplasmic staining pattern consistent with ER localization (panel 3), indicating the successful functioning of the KDEL sequence. E5/KDEL*, similar to wt E5, was also present in the Golgi (panel 2). This finding verified that a defective KDEL sequence was incapable of retaining the E5 protein in the ER and that the simple addition of six amino acids to the E5 C-terminus did not interfere with the normal localization of E5.

E5/KDEL cannot transform murine fibroblasts despite its continued expression

Since the KDEL sequence had successfully targeted E5 to an aberrant cellular location and potentially displaced it from a critical site of action, we evaluated whether E5/KDEL would retain its transforming activity. Two murine fibroblast cell lines, C127 and NIH3T3, were each transfected with 5 μg of each E5 DNA and the resultant cultures assayed for focus formation after 2–3 weeks incubation (Figure 4 and Table 1). E5/KDEL was completely defective for cell transformation. In contrast, E5/KDEL* transformed murine fibroblasts with an efficiency which ranged from 26 to 54% of wild-type in C127 and NIH3T3 cells, respectively. We have also characterized a previously reported defective ER retention signal, KDEV (Tang *et al.*, 1992b), for its ability to alter E5 transforming activity. Confirming our results with E5/KDEL*, E5 containing KDEV on the C-terminus retained transforming activity (20% of wild-type in NIH3T3 cells, comparable with that observed with E5/KDEL*) and localized to the Golgi as observed by immunofluorescence microscopy.

While we have already established that the E5/KDEL protein is stable in transiently-transfected COS cells (Figure 2), it was possible that it might be unstable in the murine cells and thereby account for its inability to induce focus formation. To evaluate this possibility, C127 and NIH3T3 cell lines were generated by co-transfecting E5, E5/KDEL and E5/KDEL* DNA's with plasmid LNCX which contains the neomycin-resistance gene. Neo^R colonies were then selected, pooled and expanded for the screening E5 protein synthesis. Cells were metabolically labelled with [^{35}S]methionine and E5 was immunoprecipitated from extracts with the antibody AU1. Proteins in this experiment were separated on gels in the presence of reducing agent, thereby generating only monomeric forms of E5. In Figure 5, E5/KDEL appears somewhat less abundant than E5/KDEL* in C127 cells (lanes 3 and 4). However, in NIH3T3 cells, E5/KDEL and E5/KDEL* are present in similar amounts, despite their absolute differences in transforming activity. Thus, protein instability cannnot account for the loss of E5/KDEL transforming activity for the following reasons: (i) E5/KDEL is expressed in NIH3T3 cells at similar levels to E5/KDEL* but does not transform, (ii) E5/KDEL is expressed in COS cells at levels similar to E5/KDEL* and (iii) E5/KDEL is expressed at identical levels in 32D cells (as determined by immunoblotting techniques) to E5/KDEL* but remains transformation-defective (unpublished data). Other reasons for the transformation defect in E5/KDEL include its potential inability to interact with the two known E5 cellular target proteins, 16K and PDGF-R, or the inability of PDGF-R to signal from the ER/*cis* Golgi.

Transformation-defective E5/KDEL protein still binds 16K V-ATPase protein

One of the major binding targets for E5 in host cells is the 16K V-ATPase protein (Goldstein and Schlegel, 1990; Goldstein *et al.*, 1991, 1992b). It is possible that the interaction of E5 with 16K could modulate the activity of the V-ATPase [which mediates the acidification of many intracellular membrane compartments (Forgac, 1989)] and thereby contribute to cellular transformation by altering growth factor receptor processing and/or degradation in host cells. Several TM E5 mutants reveal a direct correlation between their ability to transform cells and to bind 16K (Goldstein *et al.*, 1992b).

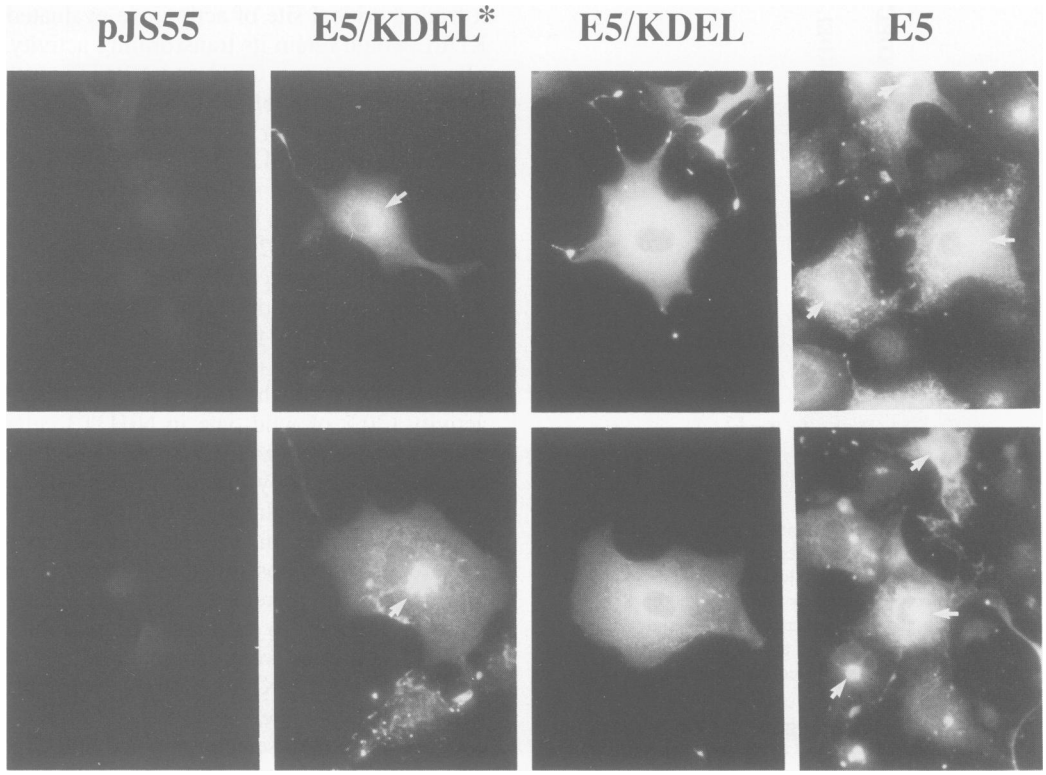

Fig. 3. Addition of KDEL to E5 alters its intracellular localization. COS cells were grown on glass coverslips and transfected with each of the E5 DNA constructs. Forty-eight hours later the cells were fixed, reacted with AU1 monoclonal antibody and evaluated by immunofluorescence as described in Materials and methods. E5 and E5/KDEL* reveal a predominant Golgi localization (see arrows). In contrast, E5/KDEL fails to accumulate in the Golgi and exhibits a reticular cytoplasmic staining pattern characteristic of the ER.

Based on immunofluorescence studies, epitope-tagged 16K protein is located predominantly in the Golgi apparatus (unpublished results). To determine whether the altered targeting of E5/KDEL to the ER would interfere with its ability to bind 16K, the immunoprecipitation studies performed above (Figure 5) were examined for co-precipitated 16K (Goldstein and Schlegel, 1990). NIH3T3 cell lines expressing E5, E5/KDEL and E5/KDEL* protein revealed the presence of 16K in all of the E5 immunoprecipitates (lanes 6–8). Since the transformation-defective E5/KDEL protein clearly bound 16K, these results demonstrate that the ability of E5 to bind 16K is insufficient for cellular transformation.

As shown in Figure 5 (lanes 1–4) and in unpublished results, E5 immunoprecipitates from C127 cells do not reveal a readily detectable 16K protein, making it impossible to evaluate the efficiency of E5/16K interaction in these cells. However, when the E5, E5/KDEL and E5/KDEL* proteins were expressed in COS cells, they exhibited similar association with endogenous 16K (data not shown), confirming the findings observed in NIH3T3 cells.

Transformation-defective E5/KDEL protein still binds PDGF-R and induces tyrosine autophosphorylation

A second protein target for E5 is PDGF-R. The transformation of fibroblasts is accompanied by the autophosphorylation of PDGF-R (Petti *et al.*, 1991; Petti and DiMaio, 1992, 1994; Nilson and DiMaio, 1993; Goldstein *et al.*, 1994), and E5 has been demonstrated to be in a physical complex with PDGF-R (Goldstein *et al.*, 1992a, 1994; Petti and DiMaio, 1992, 1994; Nilson and DiMaio, 1993) which is apparently mediated by specific interactions with the receptor TM domain (Goldstein *et al.*, 1992a; Cohen *et al.*, 1993a) rather than by its ligand binding domain (Petti and DiMaio, 1992).

To evaluate whether E5/KDEL is transformation-defective because it fails to interact with PDGF-R, we performed co-precipitation studies to evaluate E5/PDGF-R binding. NIH3T3 cells were metabolically labelled with [^{35}S]methionine, extracted and reacted first with AU1 antibody to immunoprecipitate E5 complexes. Following extensive washing, immune complexes were dissociated and then re-immunoprecipitated with anti-β-PDGF-R anti-

Table I. Transformation efficiency to E5, E5/KDEL and E5/KDEL* on two mouse cell lines

E5 construct	% WT foci production (range)[a]	
	C127	NIH3T3
Control[b]	0	0
E5	100	100
E5/KDEL	0	<0.1
E5/KDEL*	26 (25–28)	54 (40–76)

[a]Cell cultures were grown for 2–3 weeks post-transfection with 5 μg DNA as described in Figure 4. Foci were quantitated as total number on a 162 cm^2 flask. Values represent averages of three independent experiments performed in duplicate. Five micrograms of wt E5 DNA routinely produced 100–200 foci on C127 cells and 800–1000 foci on NIH3T3 cells.
[b]Control is the parent vector, pJS55, without any insert.

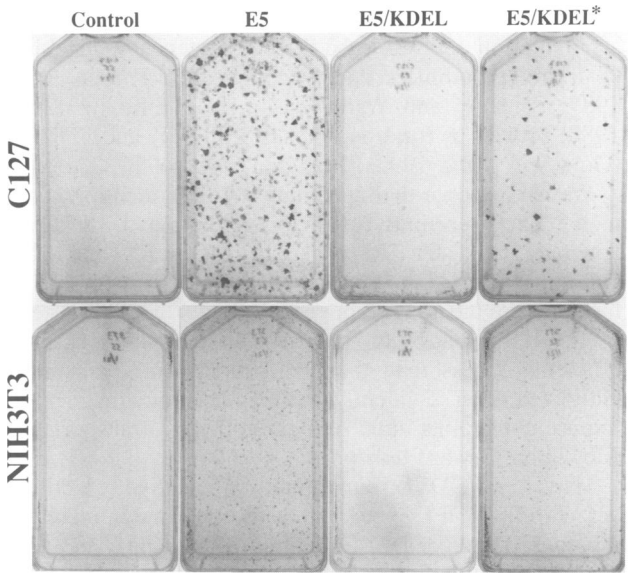

Fig. 4. E5/KDEL does not transform mouse fibroblasts. C127 and NIH3T3 monolayer cultures were transfected with each of the indicated DNA constructs, transferred into 162 cm^2 flasks and maintained for 2–3 weeks for the determination of focus formation. Although E5 and E5/KDEL* were able to transform both C127 and NIH3T3 cells, E5/KDEL was completely defective. Foci on C127 cells were larger but fewer in number than those on NIH3T3 cells.

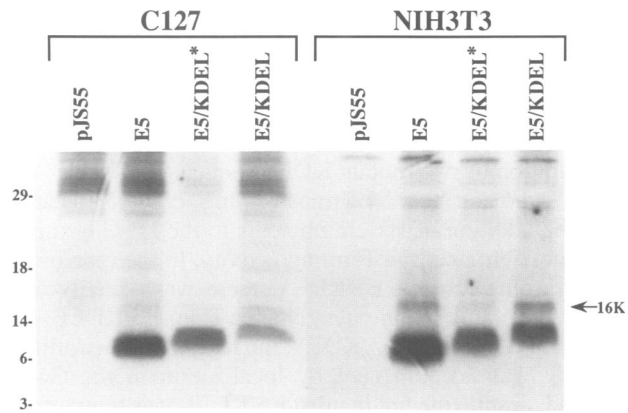

Fig. 5. E5, E5/KDEL* and E5/KDEL proteins are stable in fibroblast cell lines. E5 DNAs were co-transfected into C127 and NIH3T3 cells with the plasmid, LNCX, which confers neomycin^R. Neo^R colonies were pooled, expanded and screened for E5 expression via immunoprecipitation. Samples were separated on acrylamide gels in the presence of the reducing agent, β-mercaptoethanol. In addition to E5 protein, the appearance of a co-precipitated protein, 16K, was observed in all NIH3T3/E5 lines but could not be visualized under these conditions in C127 cells (see text). Molecular weight markers (in kDa) are listed on the left.

body. Surprisingly, the transformation-defective E5/KDEL protein associated very efficiently with PDGF-R (Figure 6). However, as might be anticipated by its ER location, E5/KDEL was associated predominantly with the immature form of PDGF-R (lane 4) whereas the wild-type and E5/KDEL* proteins associated with both immature and mature forms (lanes 2 and 3). All cell lines exhibited similar expression of both mature and immature forms of PDGF-R (see Figure 7), indicating that this differential association was not an artifact of cell line

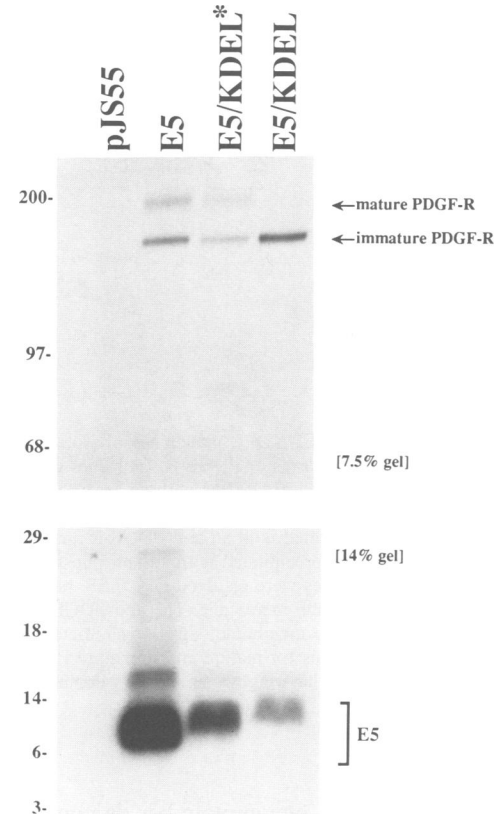

Fig. 6. E5, E5/KDEL* and E5/KDEL show similar levels of association with PDGF-R. NIH3T3 cell lines expressing each of the E5 constructs were metabolically labelled with [^{35}S]methionine as described in Materials and methods. Cell lysates were first immunoprecipitated with antibody against the epitope-tagged E5 protein (AU1). An aliquot was removed for E5 analysis on a 14% polyacrylamide gel and the remainder of the immunoprecipitate was subjected to a second immunoprecipitation using antibodies specific for PDGF-R. Proteins from the second immunoprecipitation were resolved on a 7.5% polyacrylamide gel. Positions for mature and immature PDGF-R and E5 are shown on the right. Molecular weight markers (in kDa) are listed on the left.

variation. Similar levels of binding were also observed in C127 cell lines (data not shown).

Although the E5/KDEL protein associated efficiently with PDGF-R, it was still possible that it was defective for inducing receptor autophosphorylation. To evaluate this possibility, we performed sequential immunoprecipitation/immunoblotting experiments using anti-phosphotyrosine antibodies and anti-β-PDGF-R antibodies. This would permit us to determine the level of phosphorylated PDGF-R in the E5-expressing cell lines. A portion of each lysate was used to determine the total level of PDGF-R in each cell line by immunoprecipitation and immunoblotting using the anti-β-PDGF-R antibody. As shown in the right half of Figure 7, there were no gross differences in the total levels of receptor in the cell lines. The left half of Figure 7 shows that both transformation-competent and -incompetent E5 proteins were able to stimulate the phosphorylation of both immature and mature PDGF-R to comparable levels. Not only was it unexpected that E5/KDEL stimulated PDGF-R phosphorylation equivalent to wt E5, but also that it induced phosphorylation of the mature receptor with which it was not stably associated.

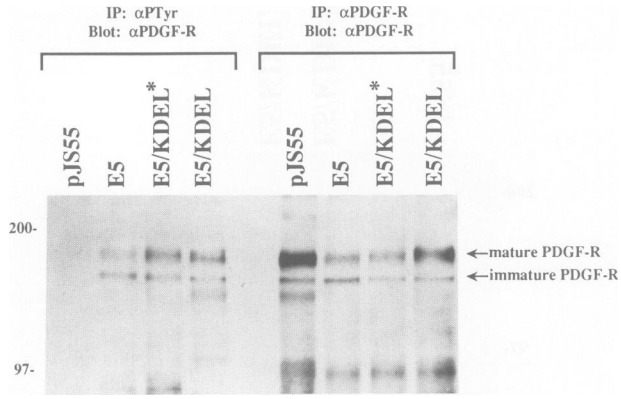

Fig. 7. E5, E5/KDEL* and E5/KDEL induce autophosphorylation of both mature and immature forms of PDGF-R. Cell lines containing the above constructs were starved in DMEM without serum for 2–3 h. Cells were then lysed in Triton X-100 lysis buffer. Ten percent of each extract was immunoprecipitated with an anti-PDGF-R antibody and the remainder was immunoprecipitated with an anti-phosphotyrosine antibody. Immunoprecipitates were resolved on a 7.5% polyacrylamide gel and transferred to a PVDF membrane. The anti-PDGF-R antibody was used to immunoblot the membrane; signals were detected by chemiluminescence. Positions for mature and immature PDGF-R are shown on the right. Molecular weight markers (in kDa) are listed on the left.

Discussion

Mutagenesis of the E5 oncoprotein has demonstrated that there are amino acid residues in both the hydrophilic C-terminal domain and the hydrophobic TM domain which are critical for transforming activity (Burkhardt *et al.*, 1987; Schlegel and Wade-Glass, 1987; Horwitz *et al.*, 1988). More recent evidence indicates that the E5 TM domain is sufficient for mediating interactions with two E5 cellular targets: PDGF-R and 16K (Goldstein *et al.*, 1992a; Andresson *et al.*, 1995) and that this domain may play a critical role in mediating the induction of PDGF-R autophosphorylation. Particularly interesting is the finding that the isolated, epitope-tagged E5 TM domain concentrates in the Golgi apparatus, indicating that the signals for targeting this organelle are present within this domain (unpublished results). To date, however, the limited number of mutants available in the E5 TM domain have not defined a mutant which is defective for Golgi localization. Thus, prior to this study, it had not been possible to evaluate whether Golgi localization was essential for cellular transformation or for initiating signal transduction through PDGF-R.

E5/KDEL contains competing 'signals' for Golgi localization and ER retention. Golgi-specific enzymes appear to be retained in the Golgi by a TM-mediated mechanism (Munro, 1991; Burke *et al.*, 1992; Colley *et al.*, 1992; Tang *et al.*, 1992a; Wong *et al.*, 1992). Retention within this organelle does not appear to rely upon specific receptor interactions but rather upon the ability of the protein's TM domain to form homo- or hetero-oligomers. Formation of such complexes is believed to interfere with the natural forward flow of proteins through the Golgi stacks and into vesicles bound for the cell surface and intracellular organelles (Nilsson *et al.*, 1991, 1993, 1994). It has also been speculated that the length of the hydrophobic TM domain may regulate protein passage through, or accumulation in, the Golgi (Bretscher and Munroe, 1993). In the case of E5, Golgi accumulation appears to be the consequence of its ability to form homodimers and oligomers (Burkhardt *et al.*, 1987; Schlegel and Wade-Glass, 1987; Horwitz *et al.*, 1988) as well as form complexes with 16K and PDGF-R (Goldstein *et al.*, 1992a; Petti and DiMaio, 1992, 1994).

We have shown that appending the ER retention signal to the E5 C-terminus blocks accumulation in the Golgi apparatus. Since the TM domain is intact in E5/KDEL, and since this domain has been shown capable of independently localizing to the Golgi (above), it appears that the retrieval of E5/KDEL back into the ER compartment is a more efficient process than the TM-mediated mechanism for Golgi retention. Evidently the E5 TM domain does not impose constraints upon retrograde protein transport as it does upon forward transport.

Why is E5/KDEL transformation-defective? A critical defect in E5/KDEL appears to be its abnormal intracellular topology. The E5/KDEL protein fails to accumulate in the Golgi apparatus as a consequence of its being recycled back into the ER and concomitantly it loses its transforming activity. Three hypotheses which can account for the loss of functional signalling by E5/KDEL are discussed below, although the last appears to be the most likely.

Addition of KDEL sequences to the E5 C-terminus directly alters protein function

One possible explanation for the inability of E5/KDEL to transform cells is that the appended KDEL sequence, as a consequence of lengthening the E5 protein, alters E5 protein conformation, thereby interfering with E5 biological activity independently of its effect on intracellular localization. This seems unlikely for two reasons. First, the addition of two mutant KDEL sequences (KDEL* and KDEV) did not abolish E5 transforming activity, indicating that the addition of six amino acids to the E5 C-terminus does not abrogate transforming activity. In the case of the transforming mutant, E5/KDEV, there was actually only one conservative amino acid change from E5/KDEL. For both E5/KDEL* and E5/KDEV, the rescue of transforming activity was accompanied by localization in the Golgi. Second, despite the finding that E5/KDEL was transformation-defective, it was still capable of binding the 16K V-ATPase protein as well as binding and inducing PDGF-R autophosphorylation, indicating that it retained its normal biochemical activities. It is possible, however, that there might be qualitative differences in PDGF-R phosphorylation induced by wt E5 and E5/KDEL which could account for their variant biological activities.

E5/KDEL does not transform cells because it cannot stably associate with mature PDGF-R

Unlike wt E5 protein, E5/KDEL does not remain stably associated with the mature form of PDGF-R during immunoprecipitation, although it does induce its autophosphorylation (Figures 6 and 7). It is possible that successful signalling through PDGF-R requires that E5 be engaged to the receptor and mediate receptor dimerization for a sufficient period of time to induce downstream signalling. Since E5/KDEL recycles from the *cis* Golgi to the ER, its premature dissociation from the receptor (following autophosphorylation) might abrogate subsequent mitogenic signalling and possibly even result in the dissociation

of the receptor back into monomeric form. This process could also generate the non-signalling, mature forms of PDGF-R which cannot be found in direct association with E5/KDEL.

It seems unlikely, however, that E5 requires mature forms of PDGF-R for inducing cell proliferation. For example, in a subclone of 32D cells which express only immature, intracellular forms of PDGF-R (as determined by molecular size and unresponsiveness to extracellular PDGF), E5 readily induces receptor autophosphorylation and cell transformation (D.Goldstein, personal communication). Thus, since E5/KDEL can bind and induce the autophosphorylation of an immature receptor which is capable of signalling (see later discussion), there must exist another blockade in the receptor mitogenic pathway.

E5/KDEL receptor complexes are not accessible to critical signalling proteins

The shuttling of E5/KDEL from the *cis* Golgi back to the ER might result in PDGF-R being translocated to a position where there is inefficient access to receptor-associated signalling molecules. Included in this list of potential intermediate substrates are GAP, Grb2/Ras, PLCγ, p85/PI3-kinase, src and syp (reviewed by Claesson-Welsch, 1994), as well as an α-adaptin-like protein with which E5 has also been shown to associate (at least *in vitro*) (Cohen *et al.*, 1993b). Thus, despite the immature receptor being associated with E5 and being autophosphorylated, it would not be able to induce a mitogenic response.

There is contradictory data in the literature concerning the ability of PDGF-R to signal from the cell interior. Reports claim either that v-*sis* must be at the cell surface in order to induce a proliferative signal through PDGF-R (Hannick and Donoghue, 1988; Fleming *et al.*, 1989) or, alternatively, that v-*sis* can function from internal sites such as the ER/*cis* Golgi (Bejcek *et al.*, 1989). The former studies utilized either suramin (which blocks PDGF/PDGF-R interactions) and/or monensin (which inhibits translocation of PDGF-R to the cell surface) to demonstrate that PDGF-R apparently needed to reach the cell surface in order to signal. However, it is clear that both of these compounds have several additional effects on the cell which might complicate the experimental findings. The latter study utilized a chimeric v-*sis* construct which, similar to our study with E5, was fused to the KDEL sequence and was apparently retained in the ER. Despite being tagged with KDEL, the v-*sis* protein was able to transform cells, suggesting that PDGF-R could signal from the ER. Extensive efforts were performed to demonstrate that the *sis*/KDEL protein was not secreted from the cell. However, sequestration of *sis*/KDEL in the ER and its absence from the Golgi were not confirmed by immunofluorescence and the translocation of even small amounts of the *sis*/KDEL protein to the Golgi might permit the effective transmission of a mitogenic signal. In our current study with an E5/KDEL chimera, potential 'leakiness' with the KDEL system does not generate a positive proliferative signal, giving more substantial confidence that PDGF-R cannot signal from the internal ER/*cis* Golgi location. Very recent studies indicate that the v-*sis* protein loses transforming activity when targeted to the early Golgi complex by the VSV-G protein cytoplasmic tail (Hart *et al.*, 1994), suggesting that *sis*/PDGF-R interactions cannot functionally signal from the proximal Golgi apparatus. These findings further substantiate our hypothesis that active E5/PDGF-R complexes cannot signal from the ER/*cis* Golgi but rather require translocation to the distal Golgi.

The E5/KDEL protein is the first reported E5 mutant that does not localize to Golgi membranes. Analysis of its biochemical and biological activities indicates that it can induce autophosphorylation of PDGF-R in the ER but that it cannot transmit a mitogenic signal from this environment. Future studies of additional E5 mutants will aid greatly in dissecting the importance of receptor intracellular topography in the regulation of signal transduction as well as defining critical regulatory proteins which are essential for transmitting a mitogenic signal from E5-associated PDGF-R.

Materials and methods

Cell culture and plasmid constructions

COS-1 and C127 and NIH3T3 cell lines were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

The expression vector PJS55 was constructed by modifying the polylinker region of pSG5 (Stratagene) and has been described previously (Sparkowski *et al.*, 1994). E5, E5/KDEL and E5/KDEL* were generated by polymerase chain reaction (PCR) using oligonucleotides synthesized on a Millipore Cyclone Plus DNA Synthesizer (see Figure 1). An oligonucleotide (5′-TTACATCTCGAGGCCACCATGGACACCTATC-CTATATACCAAATCTATGGTTT-3′) corresponding to the 5′ end of E5 codes for the first four amino acids of that molecule and contains an *Xho*I and Kozak sequence 5′ to the translational start site. In addition, the sequence coding for the epitope recognized by the monoclonal antibody, AU1, is located immediately downstream of the initiating methionine codon. Three 3′ oligonucleotides corresponding to each of the E5 constructs were also synthesized. These oligos contain complementarity to the last four or five codons of the oncoprotein with a *Bam*HI or *Bgl*II site downstream of the translational stop site: (i) wt E5, 5′-ATAGCTGGATCCTTAAAAGGGCAGACC-3′ (ii) E5/KDEL, 5′-TAGATCAGATCTTTACAACTCGTCCTTCTCAGAAAAGGGCA-GACCTGT-3′ (iii) E5/KDEL*, 5′-TAGATCAGATCTTTAAACCTCTC-CTCCTCCAGAAAAGGGCAGACCTGT-3′. Conditions for PCR synthesis were as follows: 30 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min. The plasmid containing BPV E2/E5, C88, was used as a template in these PCR reactions (Goldstein and Schlegel, 1990). All final PCR products were cleaved with *Xho*I and *Bam*HI, or *Xho*I and *Bgl*II and ligated into the *Xho*I/*Bgl*II sites of pJS55. The fidelity of all E5 constructs was verified by dideoxy sequencing.

DNA transfections

For transient expression assays, COS cells were transfected with the indicated plasmids using calcium phosphate-DNA co-precipitation as described by Graham and Van der Eb (Graham and van der Eb, 1973). One microgram of pJS55–E5 DNA was added to 9 μg carrier DNA (pUC18) in 0.5 ml 1× HEPES-buffered saline (HBS). Fifty microliters of 1.2 M $CaCl_2$ was then added slowly with mixing and incubated at room temperature for 30 min. Each sample was then added to a 50–80% confluent, 100 mm plate of cells in 5 ml fresh DMEM. Following an overnight incubation at 37°C, cells were washed with phosphate-buffered saline (PBS) prior to an addition of 2 ml of 1× HBS with 15% glycerol. Cells were incubated at room temperature for 1 min followed by three immediate washes with PBS. DMEM (10 ml) was added to plates and transfected DNA was allowed to grow for 48 h prior to immunoprecipitation analysis.

Transfections for focus assays in C127 and NIH3T3 cells were performed as described above using 5 μg each of E5 DNA and carrier DNA. Following glycerol shock, cells were allowed to grow for 3 days until confluent. Cells were then trypsinized, transferred to a 162 mm^2 flask and re-fed with 25 ml fresh DMEM every third day for 2–3 weeks.

To photograph foci, plates were stained with 1% methylene blue in 100% ethanol for 15–30 min and rinsed with water.

Transfections for generating stable lines in mouse fibroblasts were also performed as described above with modifications. E5 DNAs were co-transfected with the neomycin^R-conferring plasmid, LNCX, at a ratio of 9:1. At the time of transfer to 162 mm^2 flasks, G418 was added and maintained in the medium at a concentration of 0.5 mg/ml for NIH3T3 cells and 1 mg/ml for C127 cells. Neomycin^R colonies were allowed to form for a period of 2–3 weeks. Colonies were pooled and analysis for E5 expression was performed by immunoprecipitation as described below.

Immunoprecipitation assays

Transfected COS cells described above were analyzed for E5 protein expression by immunoprecipitation. Forty-eight hours post-transfection, cells were washed with PBS, incubated with 2 ml methionine/cysteine-free DMEM for 1.5 h and labelled at 37°C for 4 h with 0.5 mCi[^{35}S]cysteine (New England Nuclear) or 0.5 mCi [^{35}S]methionine (Pro Mix, Amersham, Arlington Heights, IL). Cells, on ice, were washed once in PBS and incubated with 1 ml of a modified RIPA buffer [20mM MOPS (morpholinepropanesulfonic acid), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate and 0.1% sodium dodecyl sulfate (SDS), pH 7.0] containing 0.1 mM protease inhibitors N-a-r-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma) and 0.5 mM phenylmethylsulfonyl fluoride (Sigma). Cells were scraped from plates, transferred to microcentrifuge tubes and vortexed for 2 min. Nuclei were removed by centrifuging tubes in an Eppendorf microcentrifuge for 1 min and transferring each supernatant to a new tube. Four microliters of the monoclonal antibody, AU1 (Goldstein *et al.*, 1992a), and 50 µl of a 1:1 suspension of protein A-Sepharose CL-4B (Pharmacia) in PBS were added to the 1 ml of extract. Tubes were rocked for 1.5 h at 4°C followed by 3×2 min washes in 1 ml of the RIPA buffer. Sepharose beads were resuspended in 30–40 µl sample buffer with or without β-mercaptoethanol, heated at 100°C for 4 min and run on 14% SDS–polyacrylamide gels. Analysis of E5 in stable fibroblast cell lines was also performed by the immunoprecipitation techniques described above. Cell lines were plated and grown to 80–90% confluency prior to incubation in methionine-free DMEM.

Analysis of the E5/PDGF-R interaction was performed by a double immunoprecipitation technique. The first immunoprecipitation was performed using the AU1 monoclonal antibody to isolate E5 protein complexes. Following two washes with RIPA buffer, the immunoprecipitates were resuspended in 1 ml RIPA from which 150 µl was aliquotted into a new microfuge tube. This sample was pelleted and resuspended in 30 µl sample buffer and subsequently run on 14% polyacrylamide gels for E5 analysis. The major portion of the sample from the first precipitation was pelleted and resuspended in 200 µl solubilization buffer (50 mM triethylamine-Cl pH 7.4, 100 mM NaCl, 2 mM EDTA pH 7.4, 0.4% SDS, 2 mM β-mercaptoethanol) and heated for 2 min at 100°C. Tubes were cooled on ice, 4 µl 0.5 M iodoacetamide was added and samples were centrifuged for 10 s. Supernatants were placed in a new microfuge tube with 50 µl 10% Triton X-100, 50 µl protein A-sepharose and 5 µl β-type PDGF-R-specific, rabbit polyclonal serum, 06–131 (Upstate Biotechnology Inc., Lake Placid, NY) added and incubated by rocking at 4°C for 1 h. Samples were washed once with PBS, 30 µl sample buffer was added and proteins were analyzed on 7.5% polyacrylamide gels. All gels derived from metabolic labelling were fixed in 30% methanol, 10% acetic acid for 10 min, treated with Enlightning (New England Nuclear) for 30 min, dried and exposed to Kodak XAR-5 film for 1–14 days at –70°C.

Immunoblotting analysis

For each cell line, three 150 cm plates of 90–95% confluent cells were washed twice in PBS and incubated in serum-free DMEM for 2 h at 37°C. Prior to harvesting, cells were washed once in PBS containing 100 mM Na_3VO_4. Cells were then extracted in 1 ml of a lysis buffer containing 1% Triton X-100, 50 mM HEPES pH 7.5, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM $NaPP_i$, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 10 µg/ml aprotinin (Boehringer Mannheim) and 10 µg/ml leupeptin (Boehringer Mannheim). Procedures for immunoprecipitation were similar to those described above. For standardization of the amount of receptor from each sample, 10% of each lysate was aliquotted for immunoprecipitation with 1 µl anti-β PDGF-R (06–131, UBI) antibody described above. The remainder of each lysate was immunoprecipitated with 5 µl anti-phosphotyrosine antibody G410 (Upstate Biotechnology, Inc.) for 2 h. Immunoprecipitates were washed three times with lysis buffer, solubilized in 30 µl sample buffer and boiled for 4 min. Proteins were then separated on 7.5% polyacrylamide gels at 230 V, followed by overnight transfer to Immobilon-P transfer membranes (Millipore, Bedford, MA) in Tris-glycine buffer with 20% methanol at 25 V. Immunoblotting was performed using a Tropix (Bedford, MA) Western Light protein detection kit and procedures described by the manufacturer. The anti-β PDGF-R antibody (06–131, UBI), at a dilution of 1:500, was used for all blots. Membranes were exposed to film for 1–10 min.

Immunofluorescence assays

COS cells were grown on glass coverslips and transfected when 60% confluent with the pJS55 constructs as described above. Forty-eight hours after glycerol shock, cells were washed twice in PBS, fixed for 20 min in PBS containing 3.7% formaldehyde and washed again three times in PBS. Coverslips were incubated for 20 min in 10% normal goat serum (NGS) and 0.1% saponin in PBS followed by two washes in PBS. Cells were then incubated for 1 h with the antibody AU1 that was diluted 1:100 in PBS containing 10% NGS/0.1% saponin. Coverslips were washed three times with PBS, incubated for 1 h with rhodamine conjugated to goat anti-mouse antibody (Jackson Labs, Bar Harbor, ME) and washed again three times in PBS. Coverslips were mounted on slides with Fluoromount (Pan-Data, Rockville, MD) mounting solution. Cells were observed and photographed with a Zeiss Axioskop inverted fluorescence microscope.

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