JOURNAL OF VIROLOGY, July 1991, p. 3481–3488
0022-538X/91/073481-08$02.00/0

Vol. 65, No. 7

Bovine Papillomavirus Type 1 Alters the Processing of Host Glucose- and Calcium-Modulated Endoplasmic Reticulum Proteins

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Received 5 December 1990/Accepted 1 April 1991

We have previously characterized five proteins induced by the presence of the E2 open reading frame (ORF) region of bovine papillomavirus type 1 (BPV-1) in C127 mouse fibroblasts (R. M. Levenson, U. G. Brinckmann, M. K. O'Banion, E. J. Androphy, J. T. Schiller, F. Tabatabai, L. P. Turek, K. Neary, M. T. Chin, T. R. Broker, L. T. Chow, and D. A. Young, Virology 172:170–179, 1989). By specific immunoprecipitation, we now find that one of the papillomavirus-associated proteins (pvp1) is a highly glycosylated form of glucose-regulated protein 100 (grp100), a major constituent of the endoplasmic reticulum. A second set of pvps (2, 3, and 4) are shown to be related precursors of another protein already present in C127 cells (protein B). Based on their induction by the calcium ionophore A23187 and their positions on giant two-dimensional gels, we have tentatively identified pvp2, -3, and -4 and B as forms of calcium-regulated protein 55, another constituent of the endoplasmic reticulum (D. R. J. Macer and G. L. E. Koch, J. Cell Sci. 91:61–70, 1988). The mechanism by which BPV-1 brings about these changes is not yet defined; however, it is unlikely to involve calcium level perturbations or transformation per se, since ionophore treatment changes other proteins in C127 cells not seen with BPV and the papillomavirus-associated proteins are found in nontransformed cells harboring the E2 ORF region. Furthermore, the BPV changes are not associated with increased grp mRNA levels, as occurs in ionophore-treated cells. Rather, it appears that BPV-1 somehow retards the normal processing of these resident endoplasmic reticulum proteins that are believed to serve as critical regulators of host protein processing and assembly.

Papillomaviruses are small double-stranded DNA viruses which cause a variety of epithelial lesions in many species. Several human papillomaviruses are intimately associated with anogenital carcinomas (reviewed in reference 50). All papillomaviruses are uniquely dependent on the host: they show specific species and tissue tropism (42); they require the complex system of differentiating epithelium to complete their life cycle (37); and their transforming genes act by usurping normal cell growth regulation mechanisms. Specifically in regard to transformation, the E6 and E7 oncogenes, believed to be involved in the initiation of human cervical carcinomas, seem to act by binding to suppressor gene products (9, 47), whereas the major transforming gene (E5) of bovine papillomavirus type 1 (BPV-1) appears to be a constitutive activator of several growth factor receptors (26).

In order to better understand the interactions between papillomaviruses and their host cells, we had previously characterized virus-associated changes of cellular proteins in BPV-1-transformed mouse fibroblasts (C127 cells) by high-resolution giant two-dimensional gel electrophoresis (22, 23). In these studies, a set of five new proteins were detected in transformed cells (papillomavirus-associated proteins [pvps] 1 through 4 and p62), and their appearance was shown to be associated with the presence of the E2 open reading frame (ORF) region of BPV-1 (22, 30). Although the E2 ORF encodes a set of *trans*-regulatory proteins for papillomavirus transcription (17, 18, 39), at least some of the cellular protein changes we observed seemed to arise from alterations in protein processing, as opposed to direct E2 transcriptional regulation of cellular genes (30).

Here we identify one and tentatively three other BPV-associated proteins as forms of resident endoplasmic reticulum (ER) proteins that arise by processing alterations. These effects of BPV are shown to be specific and distinct from the effects of calcium ionophore, an agent that also alters ER proteins. The selective alteration of these reticuloplasms by BPV represents a potentially important virus-host interaction, since these proteins are believed to play critical roles in the processing and assembly of other host proteins (1, 27, 31).

MATERIALS AND METHODS

Cells and cell culture. C127 cells (19), BPV-1-transformed C127 cells (ID13 [19]), and the C59-3881 cell line (48) were obtained from Peter Howley. Other BPV-1-transformed lines (142.6 [36], E6oc1, E7oc2 [29], and XL3-2 [8]) were obtained from Dan DiMaio. Cells were propagated in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) without antibiotics and were monitored for mycoplasma contamination by Hoechst 33258 staining (4).

Metabolic labeling of cellular proteins and gel analysis. All experiments were carried out on exponentially growing, subconfluent (60 to 80%) monolayers, usually in six-well plates (Falcon; 35-mm-diameter wells). Cells were labeled in 0.6 ml of DMEM without methionine (GIBCO) plus 10% fetal bovine serum and either 150 μCi of [^{35}S]methionine (>1,000 Ci/mmol; New England Nuclear) or 200 μCi of Tran^{35}S-label (>1,000 Ci/mmol; ICN) per ml for various lengths of time. Monolayers were rinsed twice with ice-cold DMEM with methionine prior to lysis in 200 μl of A8 lysis buffer (9.5 M urea, 2% [wt/vol] Nonidet P-40, 2% [wt/vol] ampholines [LKB; 1.6% pH range 5 to 8; 0.4% pH range 3.5 to 10], 5% [wt/vol] 2-mercaptoethanol). Incorporation of

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label into proteins was determined by trichloroacetic acid precipitation. Details of the giant two-dimensional electrophoresis system have been described previously (49). Tunicamycin (mixture of A, B, C, and D isomers; Sigma) was prepared as a stock solution (2 mg/ml) in 50% methanol. The calcium ionophore A23187 (Calbiochem) was used at a concentration of 5 μM from a 2.5 mM stock in ethanol. Cycloheximide (Sigma) was used at a concentration of 25 μM from a 100× stock in water. This level inhibited trichloroacetic acid-measured protein synthesis by >97% within 15 min. Control cultures received appropriate amounts of solvents.

Immunoprecipitation. Purified anti-grp100 rat monoclonal antibody (9G10 [10]) was a gift of Dean Edwards, University of Colorado Health Sciences Center. Cells in a 10-cm plate, labeled as described above for 1 h, were rinsed twice with ice-cold DMEM and then suspended by scraping in an immunoprecipitation buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% [vol/vol] Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] [7]) containing 0.25 mM phenylmethylsulfonyl fluoride. After 5 min at room temperature, the lysate was clarified by centrifugation in a microfuge (15,000 × *g* for 15 min at 4°C). Purified anti-grp100 antibody (10 μg/100 μl of lysate) or normal rat serum (0.1%) was added to the lysate and allowed to incubate for 3 h at 4°C. Immune complexes were precipitated by incubating with biotinylated rabbit anti-rat immunoglobulin G (IgG; Vector Laboratories) followed by incubation with prewashed streptavidin-agarose (Bethesda Research Laboratories [BRL]). The pelleted beads were washed four times with immunoprecipitation buffer and then resuspended in A8 lysis buffer for 30 min at room temperature prior to two-dimensional gel electrophoresis.

Peptide analysis. Individual gel spots identified on fixed, dried gels by autoradiography were excised. These spots were rehydrated with 125 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.1% SDS for 30 min, directly in the wells of 1.5-mm-thick polyacrylamide gels, and then subjected to digestion with either 5 or 500 ng of staphylococcal V8 protease (Miles) (6). The peptides were separated on a linear polyacrylamide gradient (10 to 25%) gel with high-Tris buffer to maximize resolution (12).

RNA preparation and Northern (RNA blot) analysis. Total RNA was isolated from cells grown in 15-cm plates by lysis in guanidinium isothiocyanate followed by centrifugation through a cesium chloride cushion (5), or from cells grown in 10-cm plates by purification from cytoplasmic extracts (13) (used only in ionophore experiments). RNA concentrations were determined by A_{260} measurements. RNA samples (10 μg) and molecular weight markers (RNA ladder; BRL) were subjected to formaldehyde-agarose gel electrophoresis (35) and visualized by ethidium bromide staining. After electrophoresis, RNAs were transferred to nylon membranes (Duralon; Stratagene) by overnight capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate).

Membranes were prehybridized for 1 h at 42°C in 50% deionized formamide–2× SSC–50 mM $NaPO_4$ (pH 6.5)–5× Denhardt's solution (50× Denhardt's solution is 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 1% Ficoll 400)–1% SDS–150 μg of sonicated and denatured salmon sperm DNA per ml. DNA probes labeled with [^{32}P]dCTP by random priming (11) (kit from Boehringer Mannheim) to a specific activity >10^9 cpm/μg were added directly to the prehybridizing solutions (3 × 10^6 cpm/ml) and allowed to hybridize overnight at 42°C. After hybridization, the membranes were washed two times for 5 min at room temperature in 2× SSC–0.1% SDS, two times for 30 min with the same solution at 65°C, and then for 30 min at room temperature with 0.1× SSC. After air drying, the filters were exposed to Kodak XAR film with intensifying screens. cDNA probes for grp78 and grp100 (p3C5 and p4A3, respectively [21]) were a gift from Amy Lee, University of Southern California Medical School.

RESULTS

Papillomavirus-associated protein 1 (pvp1) is a highly glycosylated form of glucose-regulated protein 100 (grp100). We have previously characterized a set of six cellular proteins that are induced in BPV-1-transformed mouse C127 cells (23). Five of these are associated with the presence of the E2 ORF region even in the absence of transformation (22, 30). These proteins, identified in Fig. 1, are designated pvp1 (110 kDa, pI 5.2); pvp2, pvp3, and pvp4 (55 to 60 kDa, pI 4.8); and p62 (62 kDa, pI 6.3). The heterogeneous nature (better appreciated in Fig. 2) and location of pvp1 immediately above two other proteins suggested that pvp1 was a modified version of these two proteins. Indeed, peptide analysis of pvp1 and the two lower proteins revealed similar patterns of peptide fragments (30).

In the work reported here, we found that when cells were metabolically labeled in the presence of various concentrations of tunicamycin, an inhibitor of N-linked glycosylation, pvp1 decreased in abundance with increasing concentrations and, at 10 μg/ml, disappeared entirely (Fig. 2). The larger of the two proteins immediately below pvp1 (upper arrow) also disappeared, leaving just the lowest form. Tunicamycin, at concentrations which inhibit the synthesis of pvp1, also caused changes in other proteins (Fig. 2, larger arrowheads near the bottom), which by inference we assume to be proteins that are modified by glycosylation. Importantly, no changes in pvp2 through pvp4 (Fig. 2) or p62 (not shown) were visible in tunicamycin-treated cells, indicating that changes in glycosylation do not account for their appearance in E2 ORF-containing cells.

Glucose-regulated protein 100 (grp100) has been identified on two-dimensional gels as a glycoprotein (46) with a location similar to that of pvp1. Thus, we hypothesized that pvp1 represents a modified form of glucose-regulated protein 100. Using a rat monoclonal antibody for grp100 (9G10), we were able to specifically immunoprecipitate pvp1 and the two lower-molecular-weight forms from labeled cell lysates (Fig. 3). In this experiment, the lysate was not precleared with the secondary antibody and streptavidin-agarose, a step that eliminates nearly all the nonspecific proteins seen in the figure. Combined with our previous demonstration of the relatedness of pvp1 and the lower two spots by peptide mapping (30), the tunicamycin and immunoprecipitation experiments reported here demonstrate that pvp1 is a highly glycosylated form of grp100.

pvp1 is a rapidly turned-over form of grp100 in C127 cells. Two-dimensional gel analysis of pulse-labeled (15 min) C127 proteins revealed a heterogeneous set of proteins whose appearance and position were identical to those of pvp1 in ID13 cells (Fig. 4). In C127 cells, pvp1 was unstable, rapidly disappearing over a 1-h chase period (Fig. 4), and thus it was not seen in these cells labeled for longer periods of time. In contrast, pvp1 was stable in BPV-containing cells (Fig. 4), with a measured half-life exceeding 6 h (30).

pvp2, -3, and -4 represent processed intermediates of a protein already present in C127 cells. The finding that pvp1

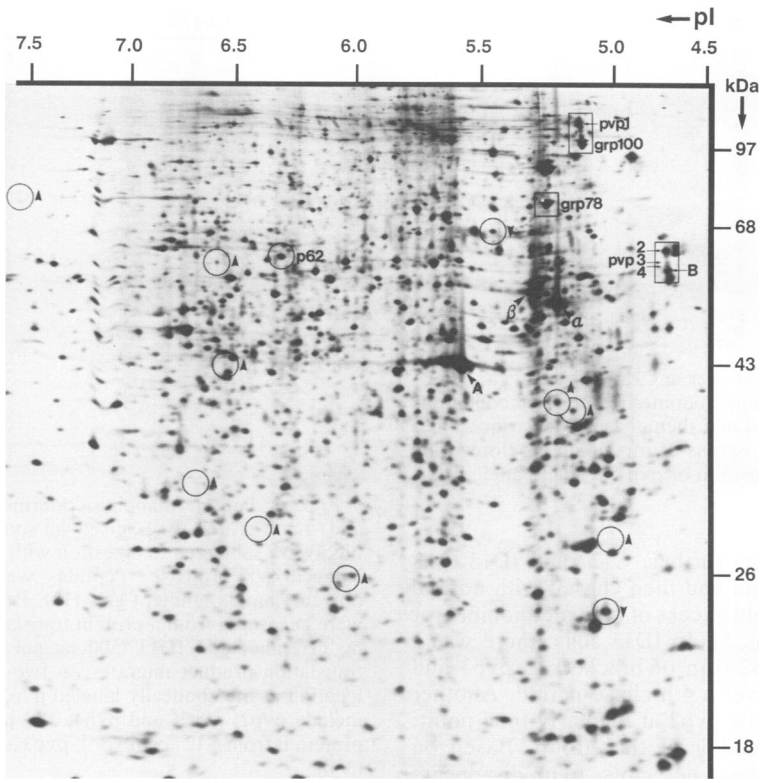

FIG. 1. Giant two-dimensional gel of metabolically labeled proteins in BPV-1-transformed cells (ID13). Subconfluent ID13 cells were labeled with Tran^{35}S-label for 1 h prior to lysis, and proteins were separated by charge in the first dimension, followed by size separation on a 10 to 16% polyacrylamide gradient gel. Five proteins not normally present in C127 cells which are associated with the presence of the E2 ORF region of BPV-1 are indicated (pvp1, -2, -3, and -4 and p62). Other labeled proteins include grp78 and grp100; actin (A); α- and β-tubulin (α, β); and proteins (or their locations) altered by ionophore treatment (circled, with arrowheads indicating induction [▲] or repression [▼]).

was an altered form of a protein found in normal C127 cells suggests that the other papillomavirus-associated changes might arise from protein processing changes. In particular, the appearance of pvp2, -3, and -4 on two-dimensional gels (proteins of the same pI, varying slightly in molecular weight) makes it likely that they are related. This was explored in two ways. We had previously observed that pvp2 was more prominent in experiments with short-term protein labeling (30 min) but that pvp3 and pvp4 were the predominant forms in longer labeling experiments (3 h) (29a).

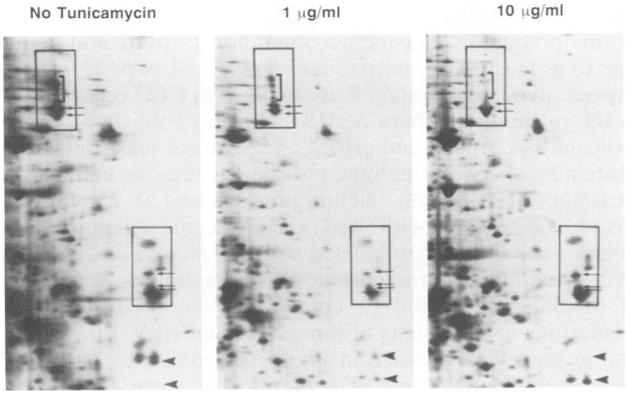

FIG. 2. Tunicamycin, an inhibitor of protein glycosylation, blocks the synthesis of pvp1. ID13 cells were treated with various concentrations of tunicamycin for 2 h and labeled with [^{35}S]methionine during the last hour of exposure. A portion of each two-dimensional gel is shown. The brackets in the upper boxes indicate pvp1, and the arrows point to two related proteins. The arrows in the lower boxes indicate pvp2, -3, and -4, respectively, from top to bottom. The arrowheads indicate glycoproteins that change with tunicamycin treatment. The gel represented by the middle panel contained less radiolabeled protein and thus appears fainter.

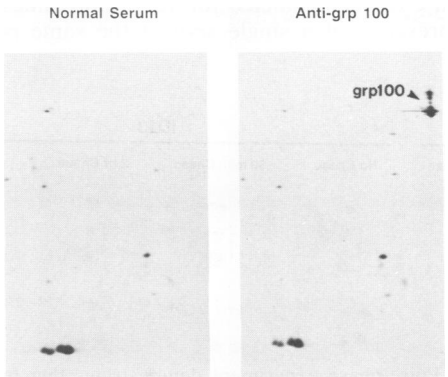

FIG. 3. pvp1 is immunoprecipitated by an anti-grp100 antibody. Lysates of methionine-labeled ID13 cells were incubated with either normal serum or a purified monoclonal antibody (9G10) specific for grp100. Examination with both one- and two-dimensional gel electrophoresis revealed a single group of proteins corresponding to pvp1 and a pair of proteins of lower molecular weight that were specifically immunoprecipitated.

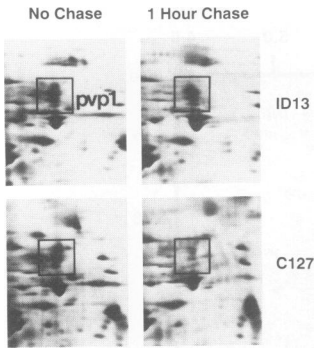

FIG. 4. pvp1 is rapidly turned over in C127 cells. C127 and ID13 cells were labeled for 15 min in methionine-free medium containing 250 μCi of Tran^{35}S-label per ml and then chased for various times with normal medium (650-fold excess of methionine) before being lysed. The box indicates the position of pvp1 in each panel.

To analyze these phenomena further, C127 and ID13 cells were pulse-labeled for 15 min and then chased with normal medium (containing a 650-fold excess of cold methionine) for various lengths of time (Fig. 5). In ID13 cells, there was a clear progression from pvp2 (top of bracket) to pvp3 and pvp4 (bottom of bracket) over a 4-h chase period. Another spot, seen immediately below pvp2 at the early time point, may represent a fourth member of this group. Based on densitometric scanning of these gel spots, pvp2 disappears with a half-life of 90 min and pvp3 and pvp4 accumulate to 50% of their maximum intensity (at 4 h) in about 45 min.

In another series of experiments, excised gel spots representing pvp2, -3, -4, and several other proteins in their general vicinity were subjected to digestion with staphylococcal V8 protease, and the peptides generated were compared by one-dimensional polyacrylamide gel electrophoresis. As shown in Fig. 6, the peptide patterns for pvp2 and pvp3-pvp4 (these spots were excised together) were very similar with two different concentrations of protease. Furthermore, this peptide pattern was also obtained for the protein immediately below pvp4. This protein, indicated as B in Fig. 1 and 5, is present in C127 cells that do not contain BPV-1 genes. The relatedness of these proteins was also established by the observation of a faint spot at the position of pvp2 in C127 cells labeled for very short times (15 min) and the presence of a single spot at the same position in

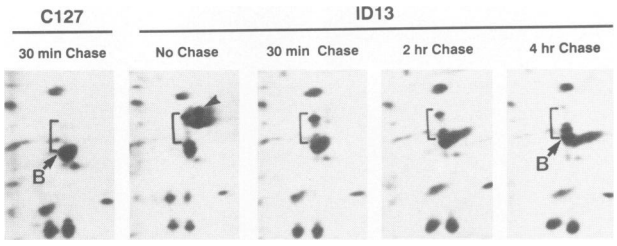

FIG. 5. Pulse-chase experiment demonstrates that pvp2, pvp3, and pvp4 are related. Cells were pulse-chased as described in the legend to Fig. 4. In ID13 cells, pvp2, pvp3, and pvp4 appear from top to bottom, respectively, within the brackets. These proteins are not seen in C127 cells (first panel). The position of protein B is indicated in the C127 panel and in the last panel. A very rapidly turning over (or secreted) protein (half-life estimated to be <5 min) is indicated by an arrowhead in the "no chase" ID13 panel.

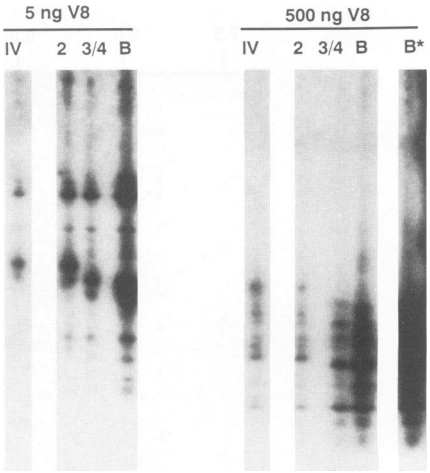

FIG. 6. Peptide mapping confirms the relatedness of pvp2, -3, and -4 and protein B. Excised gel spots, identified by autoradiography, were subjected to digestion with two concentrations of staphylococcal V8 protease. Peptides were separated on 10 to 25% polyacrylamide gradient gels (12). Peptides in the lanes labeled IV were generated from a protein translated in vitro from both C127 (5 ng per lane) and ID13 (500 ng per lane) mRNAs. This in vitro translation product migrates on two-dimensional gels in the same location as metabolically labeled pvp2 in ID13 cells. Other samples include pvp2, pvp3, and pvp4, and protein B from ID13 cells and protein B from C127 cells (B*). pvp3 and pvp4 were excised together (3/4).

two-dimensional gels of the in vitro translation products of polyadenylated RNA isolated from either ID13 or C127 cells (in this area, no differences in any in vitro translation products were observed between the two cell lines; data not shown). The V8 protease digestion pattern for this in vitro-translated protein from either C127 or ID13 cells was identical to that for pvp2 (Fig. 6). From this evidence we conclude that pvp2 is a primary protein product that is quickly converted, most likely by specific proteolytic clipping, to protein B in C127 cells. Somehow the presence of the BPV-1 E2 ORF region dramatically alters the processing of this precursor, causing accumulation of pvp2 and giving rise to at least two intermediates, pvp3 and pvp4.

pvp1, pvp2, and protein B are induced in C127 cells exposed to the calcium ionophore A23187. Several published reports indicate that grp100 and grp78, as well as at least one other protein localized in the lumen of the ER, can be induced by treatment of cells with calcium ionophores (20, 25, 46). We treated C127 cells with A23187, a specific calcium ionophore, for 4 h and then labeled cells for 30 min or 2 h either with or without continued ionophore treatment. Regardless of the labeling period or conditions, we observed some similarities in the effects of ionophore and BPV-1 on grp100 and protein B. As shown in Fig. 7, ionophore-treated C127 cells accumulated a set of proteins that were remarkably similar to pvp1 and demonstrated a clear increase in the amount of synthesized, unglycosylated grp100 (protein of lowest molecular weight). Furthermore, two proteins induced in C127 cells by ionophore treatment migrated in the same vicinity that pvp2 migrates in gels of ID13 cells, and there was an increase in the level of protein B. We also observed an induction of grp78 (Fig. 7), which on reexamination of two-dimensional gels appeared to be induced in ID13 cells.

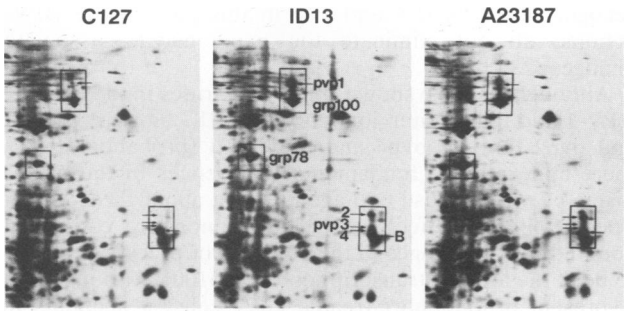

FIG. 7. Calcium ionophore treatment of C127 cells induces pvp1, pvp2, and protein B. Ionophore-treated C127 cells were exposed to 5 μM A23187 for 4 h prior to a 30-min metabolic labeling. Arrows in each panel indicate proteins marked in ID13 panel.

Despite these changes, it is important to note that at least 10 other ionophore-induced changes in cellular proteins were observed that were not seen in comparisons of C127 and ID13 proteins (the positions of these proteins are indicated in Fig. 1, and three [two inductions and one repression] are shown in Fig. 8). Furthermore, another papillomavirus-associated protein, p62, was not observed in ionophore-treated C127 cells (Fig. 8). Finally, relative to C127 cells, ID13 cells did not show a large increase in protein B, nor did we detect pvp3 or pvp4 in C127 cells exposed to A23187 (Fig. 7). These differences suggest that the mechanism by which pvp2 through pvp4 arise in BPV-containing cells is inherently different from the induction of this protein by calcium ionophore treatment. Together, these results indicate that BPV is not simply changing calcium distributions, at least not in the same way that the A23187 ionophore does.

Prevalence of grp100 and grp78 mRNAs in C127 cells harboring BPV or treated with A23187. Calcium ionophores are known to strongly induce the expression of the grp100 and grp78 genes (34). In order to establish whether the appearance of the papillomavirus-associated proteins was also associated with induction of the glucose-regulated mRNAs, we probed Northern blots of total RNA from a variety of cell lines and from A23187-treated C127 cells with labeled grp100 and grp78 cDNAs. In particular, we examined RNAs from both transformed and untransformed cell lines known to contain the pvp proteins. As shown in Fig. 9,

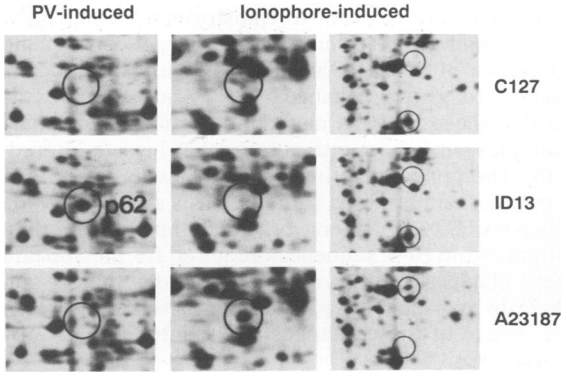

FIG. 8. p62 is not induced by calcium ionophore treatment of C127 cells and several ionophore-modulated proteins are not present in ID13 cells. The proteins that change are circled. PV, papillomavirus. Cells were labeled as described in the legend to Fig. 7.

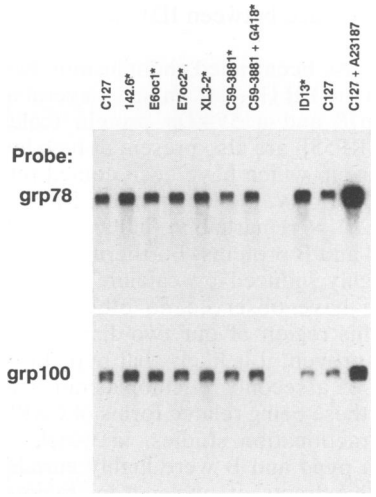

FIG. 9. grp78 and grp100 mRNA levels in cell lines exhibiting pvps. Equivalent amounts of total RNA (5 μg) were electrophoresed in parallel in agarose-formaldehyde gels, blotted, and probed with cDNA clones for grp78 and grp100. Asterisks mark cell lines containing pvps. Ethidium bromide staining of the gels revealed similar intensities for rRNA bands in all lanes.

both grp mRNAs were highly induced after 4 h of ionophore treatment. However, they were not induced above C127 levels in many of the cell lines exhibiting pvp changes (including C59-3881, a nontransformed line containing the E2 ORF expressed from the simian virus 40 early promoter [48]). In a few cases, BPV-transformed cell lines (ID13 and 142.6) did show increased levels of grp78 mRNA, though these levels did not approach those seen in cells treated with ionophore. Thus, while the grp mRNAs are expressed at modestly higher levels in some pvp-containing cells, the increased mRNA levels are not directly related to the appearance of the pvps. Again, the data suggest that changes in calcium are unlikely to account for the more selective effects of BPV on protein processing.

DISCUSSION

We have established that four of our previously observed changes in cellular proteins associated with the presence of the E2 ORF region of BPV-1 arise from alterations in the processing of two calcium ionophore-induced proteins. By using an inhibitor of glycosylation and immunoprecipitation, pvp1 appears to represent a highly glycosylated form of glucose-regulated protein 100. The transient appearance of pvp1 in pulse-labeled C127 cells and the lack of a charge difference between the three identified forms of grp100 suggest that pvp1 may simply represent a high-branched mannose intermediate in the normal processing of grp100. However, we have not been able to demonstrate an accumulation of pvp1 in C127 cells exposed to castanospermine or 1-deoxymannojirimycin, specific inhibitors of the enzymes that initiate the conversion of N-linked high-mannose core glycoproteins to their mature form (data not shown). Regardless of the relationship of pvp1 to mature grp100, the accumulation of pvp1 in BPV-containing cells is a specific BPV effect on this protein, since at least 10 other glycoproteins (identified by two-dimensional gel separations of proteins synthesized in the presence and absence of tunicamy-

cin) were unchanged between ID13 and C127 cells (data not shown).

grp100 has also been called endoplasmin, based on its high abundance in the ER (14, 24). In fact, several other proteins, including grp78 and a 55-kDa protein (calcium-regulated protein 55 [CRP55]) are also present at high levels in the ER and form a group which have been coined reticuloplasmins (15, 25). Interestingly, the location of CRP55 on two-dimensional gels (25) is remarkably similar to that of the pvp2 through pvp4 and B proteins. Furthermore, like pvp2 and B, CRP55 is highly induced by calcium ionophore treatment (25), and we have observed no other ionophore-induced proteins in this region of our two-dimensional gels. Thus, although not proven, it is likely that pvp2 through pvp4 and B are forms of a second reticuloplasmin, CRP55. Further evidence for these being related forms of CRP55 comes from subcellular fractionation studies, in which we found that pvp2 through pvp4 and B were highly enriched in low-salt Nonidet P-40 extracts of nuclei from hypotonically lysed cells (data not shown). grp78 and grp100 are known to be resident ER proteins and are also highly enriched in this fraction. Interestingly, no differences were observed between C127 and ID13 cells in the subcellular distributions of grp78, grp100, or protein B, and all of the BPV-associated forms (pvp1 through pvp4) appear in the same fraction.

The reticuloplasmins are hypothesized to function as ER traffic regulators (reviewed in reference 31). This is based in part on the finding that grp78 is identical to an immunoglobulin heavy-chain binding protein (BiP [27]). Sequence comparisons of grp100 and grp78 (BiP) reveal a conserved set of carboxy-terminal amino acids (KDEL) which confer a signal for retention in the ER (28), and a KDEL-binding protein has recently been characterized (44). Several reticuloplasmins (including grp100 and CRP55) readily bind calcium (25). Calcium ionophore treatment not only induces the synthesis of these proteins (known to be at the level of transcription for grp100 and grp78 [34]), but also leads to their transient secretion from cells (1).

How might the presence of the E2 ORF region bring about changes in the reticuloplasmins? We previously showed that the papillomavirus-associated proteins were coordinately induced in all cell lines examined, implying that a single mechanism gives rise to these changes (22). As mentioned above, the changes are inherently different from ionophore-induced changes in that they appear to represent alterations of processing steps rather than increased synthesis. This was further supported by the Northern blot analysis, which showed that increased levels of grp100 and grp78 transcripts were not prerequisite for the appearance of the papillomavirus-associated proteins.

Early in this work we had hypothesized that E2 might bring about these changes through its role as a transcriptional regulator. However, our previous analyses of mutants revealed that the N-terminal transactivating domain of the E2 protein was not required for the induction of the papillomavirus-associated proteins (22, 30), and thus they are unlikely to arise through the same transactivating functions provided to viral promoters (17, 39). Although we detected no significant changes in the steady-state levels of grp78 and grp100 gene expression in pvp-containing cells, we cannot eliminate the possibility of E2 transregulation of one or more cellular proteins that have specific effects on the processing of the reticuloplasmins. It seems important to clarify that we have not yet obtained direct mutational proof that a portion of the E2 ORF is bringing about these changes; however, we found that a number of mutations that inactivate the other recognized ORFs (E4 and E5) in this part of the BPV-1 genome do not eliminate the papillomavirus-associated changes (22).

Although we have shown some similarities in the effects of BPV-1 and the calcium ionophore A23187 on pvp1 (grp100) and pvp2 through pvp4 and protein B (probably CRP55), there appear to be fundamental differences in the mechanisms by which these two agents bring about these effects. Specifically, the effects of calcium ionophore can be accounted for by changes in the amounts of precursor (known to be at the level of transcription for grp100). Although pvp1 is present in ionophore-treated C127 cells, it accounts for only a small portion of the total grp100-related proteins and appears to turn over, based on comparisons of short- and long-term-labeled material (data not shown). In contradistinction, the presence of BPV-1 results in the accumulation of a highly glycosylated grp100 without a change in transcript level and, in the case of pvp2 through pvp4, leads to an accumulation of processing intermediates (pvp3 and pvp4). A further distinction is the "selective" effect of BPV-1, whereas ionophore treatment results in at least 10 other prominent changes on our two-dimensional gels. Clearly, BPV-1 is unlikely to bring about changes by an ionophore-like alteration of calcium balance.

In addition to perturbation of intracellular calcium balance by ionophores, which induce grp78 and grp100 through an ionophore-sensitive upstream promoter region (2, 3), a number of other perturbations can bring about the induction of the glucose-regulated proteins. These include heat shock (but only in a few specific cell lines [41, 46]), glucose deprivation (33, 46), treatment with tunicamycin and other glycosylation inhibitors (33, 45) or amino acid analogs (45), transformation by Rous sarcoma virus (33, 40), infection with paramyxovirus (32), and the presence of malfolded proteins (16). It has been suggested that the underlying reason for the induction of these proteins by transformation is that the rapid growth of transformed cells results in glucose starvation (33, 38). However, this is clearly not the mechanism involved in the induction of the papillomavirus-associated proteins, since they appear in both transformed and untransformed cells harboring the E2 ORF region (22, 30).

Tada et al. (43) have reported a significant correlation between the appearance of pvp1 through pvp4 on two-dimensional gels and the degree of transformation, whereas they found no correlation between phenotype and either episomal copy number or total BPV mRNA levels. Although it is difficult to make a direct inference from their work that these proteins are relevant to transformation, our previous work demonstrates that they arise as a direct consequence of the presence of the E2 ORF gene region rather than being secondary to growth changes (30). Thus, the possibility exists that these proteins may set a portion of the stage for the complex virus-host interactions that lead to altered cell growth. The appearance of increased levels of the grps in cells transformed by Rous sarcoma virus also adds credence to this possibility. Further work, including a more detailed definition of the BPV sequences required for the appearance of these changes, will be required in order to sort out the mechanism by which these alterations arise and to determine the relevance of these changes to the interactions between host and virus.

ACKNOWLEDGMENTS

We appreciate the excellent technical support provided by Ann Colasurdo, Stephen Beck, and Michael Battaglia. We also thank

Henry Sadowski, Thomas Wheeler, Louise Chow, and Tom Broker for stimulating conversations and suggestions. Cell lines were provided by Peter Howley and Dan DiMaio.

This work was supported by grants from the J. P. Wilmot Foundation, the Council for Tobacco Research (1774 and 1587), and Public Health Service grant CA-47650 from the National Cancer Institute.

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