Human Papillomavirus E7 Enhances Hypoxia-Inducible Factor 1–Mediated Transcription by Inhibiting Binding of Histone Deacetylases

Jason M. Bodily, Kavi P.M. Mehta, and Laimonis A. Laimins

Abstract

Infection by human papillomaviruses (HPV) leads to the formation of benign lesions, warts, and in some cases, cervical cancer. The formation of these lesions is dependent upon increased expression of proangiogenic factors. Angiogenesis is linked to tissue hypoxia through the activity of the oxygen-sensitive hypoxia-inducible factor 1α (HIF-1α). Our studies indicate that the HPV E7 protein enhances HIF-1 transcriptional activity whereas E6 functions to counteract the repressive effects of p53. Both high- and low-risk HPV E7 proteins were found to bind to HIF-1α through a domain located in the N-terminus. Importantly, the ability of E7 to enhance HIF-1 activity mapped to the C-terminus and correlated with the displacement of the histone deacetylases HDAC1, HDAC4, and HDAC7 from HIF-1α by E7. Our findings describe a novel role of the E7 oncoprotein in activating the function of a key transcription factor mediating hypoxic responses by blocking the binding of HDACs.

Introduction

Human papillomaviruses (HPV) are small, nonenveloped DNA viruses that persistently infect stratified squamous epithelia. A subset of viral types are the causative agents of a variety of malignancies, including more than 99% of cervical cancers (1, 2). HPVs infect stratified epithelia and link their life cycles to the differentiation program of the host cell (3). Following entry into the basal layer, HPVs establish themselves as low copy number episomes. As basal keratinocytes divide, one daughter cell detaches from the basement membrane and begins the process of squamous differentiation. In HPV infection, viral proteins block normal cell cycle exit upon differentiation and activate expression of host DNA replication enzymes in suprabasal cells to replicate its genome (3). The oncoproteins E6 and E7 from high-risk (cancer-associated) HPVs are responsible for maintaining differentiating cells active in the cell cycle (4). These 2 proteins promote the degradation of cellular tumor suppressors: pRb family members in the case of E7 and p53 in the case of E6. Binding and degradation of pRb family members by E7 results in the release of E2F transcription factors that drive the cell into S phase. The abrogation of pRb function by high-risk E7 proteins induces a stress response leading to elevated levels of p53, which can induce apoptosis. The high-risk E6 proteins degrade p53, thus preventing apoptosis and allowing continued proliferation. In addition to these well-known activities, both proteins have a range of other targets (4), and the extent to which these additional interactions contribute to HPV-associated carcinogenesis is not fully understood. Among the additional factors bound by E7 are histone deacetylases (HDAC), which catalyze the deacetylation of histones and other transcriptional regulatory proteins (5–7). The binding of HDACs by E7 results in the activation of cellular promoters and is necessary for the differentiation-dependent phase of the virus life cycle (5, 6).

One important characteristic of both benign and malignant lesions is the promotion of angiogenesis or the formation of new blood vessels, which allows a growing lesion to access nutrients and oxygen for growth (8). Angiogenesis is triggered by hypoxia or reduced tissue oxygen levels. The cellular response to hypoxia is primarily regulated through the activity of the transcription factor hypoxia-inducible factor-1 (HIF-1; refs. 9, 10). Under normal oxygen conditions (normoxia), the HIF-1α subunit has a very short half-life due to oxygen-dependent hydroxylation and consequent degradation through the von Hippel–Lindau (VHL)/proteasome pathway. Under hypoxic conditions, reduced oxygen levels result in the accumulation of HIF-1α, which activates expression of HIF-1 target genes, including a range of proangiogenic factors and enzymes that favor glycolytic over aerobic metabolism (10).

HIF-1 is regulated by a number of factors including p53, p300/CREB, and HDACs. Although HDACs frequently inhibit transcription, HDAC activity is necessary for HIF-1 activity (11–16) and angiogenesis (17) as treatment with inhibitors...
reduces HIF-1–mediated transcription. HDAC(s) that are involved in this activation and the mechanisms responsible are controversial. HDAC1 (11), HDAC4 (14, 18), HDAC5 (18), HDAC6 (14, 15), HDAC7 (19), and SIRT-6 (20) have each been reported to bind and/or regulate HIF-1 activity. This activation has been reported to occur through a variety of mechanisms including direct deacetylation of HIF-1α (11, 14), facilitation of nuclear localization (19), increasing p300 binding to HIF-1 (16, 18, 19), or through altering interactions with HSP70/90 (15). HIF-1 can also act as a repressor of some promoters such as the cyclin D promoter, through a mechanism dependent on HDAC7 (21).

Recently, several studies have reported that HPV gene products can induce the production of angiogenic factors by infected cells (22–25). Our previous work demonstrated that cells maintaining HPV genomes show enhanced levels of HIF-1α and increased expression of HIF-1 target genes under hypoxic conditions (26). In the present study, we demonstrate that E7 is responsible for enhanced HIF-1α activity. Our studies indicate that E7 enhances HIF-1 dependent transcription by inducing the dissociation of HDACs from HIF-1α. These findings shed light on the mechanisms by which HPV contributes to tumor angiogenesis and describe a novel role of E7 in the activation of host gene expression.

Materials and Methods

Cell culture and creation of cell lines

Keratinocytes were isolated from neonatal foreskins. Keratinocytes and cell lines containing HPV genomes were cultured in E-medium NIH 3T3 J2 fibroblast feeders as described previously (27). Cells stably expressing HPV16 E7 from retroviral vectors were established by infection of keratinocytes followed by selection with G418 as described previously (28). Cell lines containing HPV episomes (HFK16 cells) were created by transfection of cloned recircularized viral DNA as described previously (29). U2OS cells were obtained from ATCC (American Type Culture Collection) and cultivated in DMEM (Invitrogen) supplemented with 10% bovine growth serum (VWR). Hypoxia was mimicked by treatment for 6 to 16 hours with 100 μmol/L deferoxamine mesylate (DFO; Sigma). Trichostatin A (300 nmol/L TSA; Sigma) was dissolved in dimethyl sulfoxide (DMSO), and cells were treated for 16 hours.

Plasmids and cloning

pcDNA HIF-1α was a gift from Eric Huang, National Cancer Institute, Bethesda, MD. pGW1 HA 18, 16, and 11 E7 were cloned by removing the 3′E7 from pHA31E7 and ligating inserts created by PCR using primers shown in Supplementary Table 1, pcDNA TapC 16E7 was created by PCR using primers OZ5′ and OZ HA Bam3′ with an HA-flag double tagged HPV16 E7 retrovirus vector (a gift from Margaret McLaughlin-Drubin, Brigham and Women’s Hospital/Harvard Medical School, Cambridge, MA) as template. pcDNA TapN 16E7 was created by PCR using 16E7 Xho frame 5′ and 16E7 Not stop 3′ as primers and pUC HPV16 as template. The product was ligated into pOZN at the Xho/Not site to create pOZN 16E7. PCR was performed again using pOZN 16 E7 using OZN 5′ Nhe and 16E7 Not stop 3′ primers and ligating into the NheI/NotI site of pcDNA 3.1(−) to create pcDNA TapN 16E7. Deletions were created by PCR using primers listed in Supplementary Table 1 and ligating into the NotI/Xhol site of pcDNA TapN 16E7. Site-directed mutagenesis was performed using the QuickChange II Site Directed Mutagenesis kit (Agilent) using the primers listed in Supplementary Table 1. pcDNA TapC 16E6 was cloned by removing E7 from pcDNA TapC 16E7 by digestion with Xhol and NotI and ligating a PCR product created using 16E6 pOZ5 and 16E6 pOZ3′ as primers and pUC HPV16 as template. All constructs were checked by sequencing before use. HPV31 E6 was expressed from pSG 31E6.

Transient transfection and Western blot analysis

Transient transfections of keratinocytes and U2OS cells were performed using polyethyleneimine (PEI; Polysciences). Whole-cell extracts were prepared 24 to 36 hours after transfection and subjected to SDS-PAGE and Western blotting or immunoprecipitation as described previously (26, 28). Antibodies used in this study are shown in Supplementary Table 2.

Luciferase assays

A luciferase reporter consisting of a trimerezed 24-mer containing 18 bp from the hypoxia response element (HRE) of the phosphoglycerate kinase promoter, HRE-TK-Luc, was a gift from Navdeep Chandel, Department of Medicine, Northwestern University, Chicago, IL. The pGL2 CA9 reporter was a gift from Stefan Kaluz, Emory University, Atlanta, GA. The pGL3 VEGF pro was a gift from Lee Ellis, MD Anderson Cancer Center, Houston, TX. One hundred thousand cells were plated per well in 6-well plates and transfected with PEI at 37°C overnight. Fresh medium was added, and cells were subsequently treated with DFO and/or TSA overnight. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), with Renilla luciferase as an internal control according to manufacturer’s instructions. Significance was determined using Student’s t test.

Quantitative real-time (RT) PCR

Total RNAs were harvested using RNA-STAT 60 (TelTest, Inc.). cDNAs were synthesized using the SuperScript First Round Synthesis System (Invitrogen), and qPCR was performed using LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480 instrument. Primers used to detect CA9, TSP-1, PGK-1, and actin (as a reference) are shown in Supplementary Table 1.

Results

E7 activation of HIF-1–dependent transcription

In previous studies, we demonstrated that cells harboring HPV genomes display an enhanced HIF-1–dependent transcriptional response to hypoxia (26). We investigated which viral protein is responsible for this effect and first examined the role of E7. For these studies, an expression vector for 16E7 was cotransfected into U2OS cells together with a luciferase reporter, HRE-TK-Luc, containing a trimerezed HRE from the
phosphoglycerate kinase promoter, and cells were treated with the hypoxia mimic DFO. We found that activity of the HIF-1 reporter was enhanced upon cotransfection with 16E7 (P < 0.01; Fig. 1A). This effect was seen only upon DFO treatment and not in normoxia (not shown). Enhancement of activity was also seen using the HIF-1–dependent carbonic anhydrase 9 (CA9) and VEGF promoters (P < 0.01; Fig. 1A).

When similar transient expression experiments were performed in human foreskin keratinocytes (HFK), a comparable enhancement in HIF-1 activity was seen (not shown).

It was next important to determine whether E6 would have an effect on HIF-1–dependent transcriptional activity. In contrast to E7, cotransfection of an expression vector for HPV 16 E6 in U2OS cells, which express wild-type (WT) p53, had no effect on transcription from the HRE or CA9 reporters (Fig. 1B). We conclude that E7 is the primary viral protein responsible for enhancing HIF-1 transcriptional activity.

To determine whether E7 alone had an effect on endogenous HIF-1 transcription in keratinocytes, transcripts from HFKs or HFKs stably expressing HPV16 E7 from an integrated retrovirus vector were examined for the levels of 3 HIF-1 target genes: CA9, TSP1, and PGK1. As shown in Figure 1C, each of these transcripts was expressed at elevated levels in 16E7 cells providing further support that E7 expression is sufficient to increase levels of HIF-1 targets in keratinocytes.

The effect of p53 on transcriptional activity
To determine the mechanism of transcriptional enhancement by E7, we asked whether p53 might be involved. p53 has been reported to inhibit HIF-1 transcriptional activity (10, 30–35) and E7 can alter the levels as well as several functions of p53 (36–40). We therefore investigated whether E7 could enhance HIF-1 activity by counteracting p53-mediated inhibition of HIF-1α. We first observed that cotransfection of a vector expressing p53 with the reporter reduced activity by approximately one half. When an expression vector for E7 was cotransfected along with p53 expression plasmids, HIF-1 activity was increased but still reduced from that seen in the absence of p53 (Fig. 1D). We conclude that E7 and p53 act independently to alter HIF-1 activity, and the effect of each is not altered by the other. This indicates that enhancement of E7 of HIF-1 activity is likely not due to an effect on p53-mediated inhibition.

Although E6 cannot directly activate HIF-1–mediated transcription, we suspected that E6 might augment HIF-1 activity in the presence of additional p53 through its ability to induce degradation of p53. To investigate this possibility, an expression vector for E6 was transfected along with the p53 vector and we observed that E6 was able to block p53-mediated inhibition. Although E6 does not directly synergize or interfere with the ability of E7 to enhance HIF-1 activity, it can overcome the repressive effects of high levels of p53 (Fig. 1D).

E7 binds HIF-1α
To determine the mechanism by which E7 enhances HIF-1–mediated transcription, we next investigated whether E7 could form a complex with HIF-1α. For this analysis, U2OS cells were transiently transfected with expression plasmids for HIF-1α and HA-tagged E7 from HPV16, 31, and 18. Cell
extracts were then immunoprecipitated with antibodies to the HA tag and screened for the presence of HIF-1α. Expression vectors encoding HA-tagged E7s from HPV types 31, 16, 18, and 11 were cotransfected overnight into U2OS cells with an expression vector for HIF-1α. Cells were treated with 100 μmol/L DFO for 6 hours or left untreated. E7-containing complexes were immunoprecipitated from total cell lysates with anti-HA antibodies. HIF-1α was detected by SDS/PAGE and Western blotting of the immunoprecipitates. B, HFK or HFK stably maintaining HPV16 genomes were treated or untreated with 100 μmol/L DFO for 6 hours and immunoprecipitation was performed from total cell lysates using anti-16E7. HIF-1α and pRb were detected in the immunoprecipitates or in total lysates by SDS/PAGE and Western blotting.

Unlike 11E7, high-risk E7s migrated slower than their predicted molecular weights, as previously observed by others (41).

To determine whether complex formation can be detected when E7 and HIF-1α are expressed at physiologic levels, we created cell lines that stably maintain HPV16 episomes by transfecting HFK with recircularized HPV16 genomes (HKFK16). Following drug selection, these cell lines were expanded in culture and found to maintain episomal HPV16 at approximately 100 copies per cell (not shown). HKFK16 cells were then treated with DFO, lysates were immunoprecipitated with antibodies directed against 16E7, and the immunoprecipitates were analyzed by Western blot analysis for the presence of HIF-1α. Consistent with results from transient transfection studies, 16E7 expressed from episomally replicating HPV16 genomes was able to coimmunoprecipitate endogenous HIF-1α (Fig. 2B). Levels of HIF-1α were increased in HPV-containing cells treated with DFO relative to those in HFK cells, in agreement with previous studies (26). As a control, we confirmed that 16E7 was able to form complexes with pRb in the same cell lines. We conclude that the interaction between 16E7 and HIF-1α occurs under physiologic conditions in cell culture.

### Binding of E7 to HIF-1α is not necessary for transcriptional activation

Several important regulatory domains of E7 have been identified through extensive functional and biochemical analyses by many groups (42; Fig. 3A). The CR1 and CR2 domains in the N-terminus are involved in pRb binding and degradation whereas the C-terminus contains motifs that bind zinc, as well as HDAC, pCAF, E2F6, and other factors. To determine which region of E7 is responsible for binding HIF-1α, we generated deletions of E7 and tested their ability to bind
HIF-1α in transient transfection/coimmunoprecipitation analyses. The plasmid pΔ1-30 contains a deletion of the first 30 amino acids of E7, which includes the pRb-binding and degradation domains. The plasmid pΔ84-98 contains a deletion of the last 14 amino acids from the C-terminus of E7, thereby disrupting the zinc-binding C-terminal domain. Following transient transfection of U2OS cells, the mutant E7Δ1-30 protein was expressed at high levels and was substantially reduced in its ability to associate with HIF-1α (Fig. 3B). This indicates that the binding site for HIF-1α in E7 maps to the N-terminal 30 amino acids. Although E7Δ84-98 was expressed at levels somewhat below that of full length E7, it formed complexes with HIF-1α at a level comparable to full length. We conclude that the C-terminus of E7 is dispensable for binding HIF-1α and that binding activity is localized to the N-terminus.

We next tested several previously characterized point mutants and small deletions of E7 for their ability to form complexes with HIF-1α. The LYCYE mutant consists of an in-frame deletion of the whole pRb-binding domain, whereas C24G and E26G are point mutations within this motif (43, 44). The L67R and C91S substitution mutations disrupt the C-terminal domain and have been shown to abrogate, among other things, ability of E7 to interact with HDACs (5). We found that all 3 mutations in the pRb-binding domain reduced the ability of E7 to associate with HIF-1α, suggesting a role for this domain in the interaction between E7 and HIF-1α. Interestingly, both mutations in the C-terminus not only retained the ability to bind HIF-1α, they showed a clear and reproducible increase in binding as compared with wild type (Fig. 4A). Furthermore, mutants in the CRI domain bound to HIF-1α as well as wild type (not shown). We conclude that the N-terminus of E7 and the pRb-binding domain in particular is responsible for binding to HIF-1α.

We next investigated if binding of E7 to HIF-1α was important for its ability to enhance transcriptional activity. Surprisingly, we found that mutants in the pRb-binding domain were as effective as wild type at activating HIF-1α transcription (P > 0.3 vs. wild type). In contrast, mutations that disrupted the C-terminal domain, L67R and C91S, were severely attenuated for transcriptional activation, and this was similar to the reporter levels seen in the absence of E7 (Fig. 4B). Therefore, even though the C-terminus is dispensable for binding of E7 to HIF-1α, it is necessary for enhancement of HIF-1α transcriptional activity by E7. These data, therefore, indicate that an activity of E7 other than binding to HIF-1α is necessary for enhancement of transcriptional activity.

Enhancement of HIF-1α activity by E7 requires HDAC activity

Because E7 binding to HIF-1α is not necessary for its activation, we considered other possible mechanisms focusing on activities mediated through the C-terminus of E7. In transient reporter assays, the ability of E7 to enhance HIF-1α transcription was reduced by the C91S and L67R mutations. These 2 mutations have been reported to block the binding of E7 with a number of cellular factors, including HDACs (5, 45) and HDACs have been implicated in enhancing HIF-1α activity (11–16). We investigated whether E7’s enhancement of HIF-1α activity requires HDAC activity by treating transiently transfected U2OS cells with TSA, a broad-spectrum HDAC inhibitor. Exposure of these cells to TSA abrogated activation of HIF-1α-dependent transcriptional activity, confirming that in our system HDACs are important for HIF-1α function (P < 0.01; Fig. 5A). Significantly, E7 had no enhancing effect on HIF-1α activity in the absence of HDAC activity in cells treated with TSA.

We next tested whether HIF-1α is itself acetylated and whether acetylation is affected by E7. Using lysates from U2OS cells transiently transfected with a HIF-1α expression vector, we performed immunoprecipitations with an antibody against acetylated lysine and then screened for HIF-1α by Western analysis. As shown in Figure 5B, HIF-1α was clearly detected by this analysis indicating that either HIF-1α itself or a protein associated with HIF-1α is acetylated. Importantly the level of acetylation was unchanged by the presence of E7. When a similar coimmunoprecipitation experiment was performed using lysates from HFKs or HFK16 cells that stably maintain HPV episomes, we found no differences in the amount of HIF-1α immunoprecipitated with anti-acetylated lysine antibody.

**Figure 4.** Mapping the HIF-1α transcriptional enhancement domain of E7. A, U2OS cells were cotransfected with the indicated 16E7 deletion construct and an expression vector for HIF-1α. Following 24 hours incubation, cells were treated with 100 μmol/L DFO for 16 hours and total lysates prepared. E7-containing complexes were immunoprecipitated with anti-HA antibodies. HIF-1α was detected by SDS/PAGE and Western blotting of the immunoprecipitates. B, U2OS cells were cotransfected overnight with the indicated E7 mutant construct and a reporter for the CA9 promoter. After 24 hours, samples were treated with 100 μmol/L DFO for an additional 16 hours. Lysates were prepared and the activities of firefly and Renilla luciferase were measured. Values were normalized to the Renilla luciferase activity in each sample, and the reporter alone with DFO was set to 1. Each point represents the mean of 6 experiments and error bars represent ± 1 SEM.
(Fig. 5C). These results indicate that E7 does not alter the level of HIF-1α acetylation and it is unlikely to be the mechanism by which E7 modulates HIF-1 activity. In addition, cell fractionation analyses indicated that E7 had no effect on the subcellular localization of either HIF-1α or HDAC1 (not shown).

E7 displaces HDACs from HIF-1α

Another mechanism to explain how association of E7 with HDACs results in HIF-1 activation could involve the modulation of HIF-1α binding to HDACs by E7. We, therefore, investigated the state of HDAC1 complexes with HIF-1α in the presence of E7. For these studies, we first transfected HIF-1α expression vectors into U2OS cells, immunoprecipitated HDAC1-containing complexes and screened for the association of HIF-1α by Western blot analysis. Consistent with previous reports, we confirmed that HIF-1α and HDAC1 form complexes in cells (Fig. 6A; ref. 11). When E7 was cotransfected along with HIF-1α, a dramatic reduction in the amount of HIF-1α bound to HDAC1 was observed. Importantly, both of the C-terminal mutants of E7, which were defective in activating HIF-1, were unable to displace HDAC1 from HIF-1α (Fig. 6A). Furthermore, the LYCYE mutant, which was able to enhance HIF-1 activity despite reduced binding to HIF-1α, was able to mediate displacement of HDAC1 from HIF-1α (Fig. 6C). We also examined the interaction of HIF-1α with 2 additional HDACs, HDAC4 and HDAC7, that have been implicated in other studies to be important in HIF-1 regulation and confirmed that both formed complexes with HIF-1α (14, 18, 19). When E7 was included in the transfections, both
HDAC4 and HDAC7 were displaced from HIF-1α in a manner dependent on the C-terminal domain of E7, similar to HDAC1. We conclude that the ability of E7 to displace HDACs from HIF-1α correlates with transcriptional enhancement by E7. Binding of HIF-1α to p300/CREB is also reported to be an important regulator of HIF-1 activity (9, 46, 47), but we did not observe any consistent changes in the association between HIF-1α and p300 or with pCAF due to the presence of E7 (Fig. 6A). It was next important to confirm that similar effects would be seen in cells that stably express E7 at physiologic levels. For this study, we immunoprecipitated HDAC1 from HFKs or HFK16 cells and then analyzed the resultant complexes for the levels of bound HIF-1α by Western analysis. Similar to our observations in transient transfections, we found that HIF-1α/HDAC1 association is markedly reduced in cells maintaining HPV16, despite increased total levels of HDAC1 (Fig. 6C). This indicates that the E7 acts to reduce the binding of HDACs to HIF-1 and this correlates with enhanced ability of HIF-1 to activate target promoters.
In summary, our studies indicate that E7 can activate HIF-1-dependent transcription by blocking the interaction of HDACs with HIF-1α in a manner dependent on the HDAC binding domain of E7. E6 primarily blocks the inhibitory effects of p53 on HIF-1 activity. These findings shed light on the mechanisms by which HPV contributes to tumor angiogenesis and describes a novel function of the E7 proteins in the viral pathogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Eric Huang, Navleesh Chandel, Stefan Kaluz, Lee Ellis, Bayar Thimmmapaya, and Margaret McLaughlin-Imbin for materials. Keya Raychaudhuri and Kyle Jamison for technical assistance; and Kathy Bundell and members of the Laimins laboratory for helpful discussion.

Grant Support

This work was supported by grants to L.A. Laimins from the National Cancer Institute (R01CA174202 and R01CA99655). This work was funded by grants from the NIH (R01CA174202 and R01CA99655). This work was funded by grants from the NIH (R01CA174202 and R01CA99655). This work was funded by grants from the NIH (R01CA174202 and R01CA99655). This work was funded by grants from the NIH (R01CA174202 and R01CA99655).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 19, 2010; revised November 23, 2010; accepted November 27, 2010; published OnlineFirst December 8, 2010.


Human Papillomavirus E7 Enhances Hypoxia-Inducible Factor 1–Mediated Transcription by Inhibiting Binding of Histone Deacetylases

Jason M. Bodily, Kavi P.M. Mehta and Laimonis A. Laimins

Cancer Res  Published OnlineFirst December 8, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-2626

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/12/08/0008-5472.CAN-10-2626.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.