The Cytoskeleton Differentially Localizes the Early Growth Response Gene-1 Protein in Cancer and Benign Cells of the Prostate

Gloria R. Mora,1 Kenneth R. Olivier,2 John C. Cheville,3 Richard F. Mitchell, Jr.,1 Wilma L. Lingle,3 and Donald J. Tindall1,4

Abstract
Prostate cancer is the most prevalent malignancy and the second leading cause of cancer mortality in men. Early growth response gene-1 (EGR-1) plays a crucial role in the development and progression of prostate cancer. The data demonstrate that EGR-1 in benign cells contains lower levels of EGR-1 located predominantly in the cytoplasm, whereas malignant cells contained higher levels of EGR-1 located predominantly in the nucleus. Benign prostate cells responded to mitogens in vitro, with increased levels of EGR-1, rapid nuclear translocation, and enhanced transcriptional activity, whereas malignant prostate cells did not exhibit the same responses and the protein remained in the cytoplasm. The central aspect of this difference is the association of EGR-1 with microtubules, which is exclusive to the benign cells of the prostate and is required for the nuclear translocation and transcriptional activity of EGR-1 in vitro. Our data demonstrate that the differences in EGR-1 between benign and malignant prostate cells extend beyond cellular levels, which was confirmed by immunohistochemistry in human tissues. Thus, we add the novel concept that microtubules regulate EGR-1 localization in benign prostate cells but not in malignant prostate cells.

Introduction
Exposure of cells to mitotic, differentiating, and/or death stimuli can lead to the rapid but transient expression of early growth response genes. Regulation at the transcriptional and post-transcriptional levels of these genes results in protein products that act as transcription factors on nuclear transport. A well-characterized member of the early growth response family, early growth response gene-1 (EGR-1; 1, 2), is a key mediator in responses to a variety of stimuli including serum (3), growth factors (4, 5), phorbol esters (5), and radiation (6, 7) in a wide spectrum of cell types. Apart from its classic role in apoptosis, differentiation, and cell growth, evidence suggests an active role for EGR-1 in tumorigenesis. The overexpression of EGR-1 inhibits the growth and tumorigenic potential of v-sis-transformed mouse NIH 3T3 cells (8), human HT-1080 fibrosarcoma cells, ZR-75-1 breast carcinoma cells, U251 glioblastoma cells, SAOS-2 osteosarcoma cells, and esophageal carcinoma cells (9, 10). In addition, EGR-1 is differentially expressed in benign cells compared with carcinoma cells in breast, brain, and lung tissues. In these tissues, cancer cells contain lower EGR-1 levels with respect to benign cells (11–13). Thus, it has been suggested that EGR-1 acts as a tumor suppressor, the levels of which are down-regulated during the transformation of normal to cancer cells (14). In contrast, we and others have shown that increased levels of EGR-1 are found in prostate cancer compared with benign prostate tissue (15, 16). Furthermore, the tumor suppressor abilities of EGR-1 have not been demonstrated in prostate cells. Indeed, ectopic expression of EGR-1 in the prostate cancer cell line PC-3 shows no tumor suppressor activity (9). Moreover, in vivo disruption of the EGR-1 gene in transgenic mouse models of prostate cancer (TRAMP and CR2-T-Ag) produces tumors with significantly slower progression from the premalignant lesion to invasive carcinoma (17). Thus, EGR-1 appears to have a unique function in the malignancy of the prostate gland. This tissue-specific difference could be due to several possibilities. The tumor suppressor activity of EGR-1 in prostate cells may be abrogated by inhibiting either its transcriptional activity or its access to target genes. Another possibility is that EGR-1 has a unique tumor-promoting role through transcriptional activation of prostate cancer-specific pathways. Because there is no sufficient evidence defining the transcriptional capability of EGR-1 in prostate cancer, and the existing data do not provide support for either option, we hypothesized that to participate in prostate tumorigenesis, the regulation of the expression and cellular localization of the EGR-1 protein must differ in cancer and benign cells of the prostate. Here, we show that EGR-1 associates with cytoskeletal components of prostate cells in a cell-specific manner. Our results demonstrate that microtubules regulate the nuclear translocation and transcriptional activity of EGR-1 in benign prostate cells but not in prostate cancer cells.
Results

EGR-1 Expression and Subcellular Localization in Prostate Cells

We have shown previously by in situ hybridization that EGR-1 mRNA expression increases progressively during prostate cancer development (16). In further analysis of EGR-1 protein expression in malignant and nonmalignant tissues, we identified intense staining in prostatic adenocarcinoma (Fig. 1A, arrows) and weaker staining in benign prostatic epithelia (Fig. 1A, arrowheads). In addition, the localization of EGR-1 staining differed between cancer and benign tissues. In cancer, EGR-1 immunostaining was less intense in nuclei relative to the high levels found in the cytoplasm (Fig. 1B-a, arrow). In contrast, a strong nuclear localization was found in the basal cells of the benign epithelium (Fig. 1, B-B and C-c, asterisks). Low levels of EGR-1 staining were detected in the secretory epithelium of benign prostatic hyperplasia (BPH) tissues (Fig. 1C), but the localization of EGR-1 was nuclear, as evidenced by the purple nuclei (Fig. 1C-c, arrowhead). These data suggest that benign and cancer tissues from the prostate differ not only in the levels of EGR-1 expression but also in intracellular localization. Therefore, because EGR-1 plays a vital role in the proliferation of prostate cancer cells, we sought to investigate the dynamics and cellular localization of EGR-1 in benign and malignant prostate cells.

To investigate the underlying cellular mechanisms that contributed to the differential expression of EGR-1 in benign and malignant prostate cells, several established cell lines were investigated. Two benign cell lines were used: BPH-1, derived from BPH tissue and immortalized with the SV40 large T antigen (18), and RWPE-1, derived from normal prostate and immortalized using the entire human papillomavirus type 18 (19). Three cancer cell lines were used: PC-3, LNCaP, and DU-145, derived from prostate cancer metastases to the bone, lymph node, and brain, respectively (reviewed in 20).

During preliminary immunolocalization studies, we noted that EGR-1 appeared to be associated with cytoskeletal structures in the benign BPH-1 cells and in the cancer DU-145 cell lines. This unusual intracellular localization of EGR-1 in prostate cells prompted further investigation using immunofluorescence and confocal analysis. In rapidly growing BPH-1 cells during the interphase (Fig. 2A-a), EGR-1 (red) colocalizes with the centrosome (arrowhead) and some microtubules (arrows). Through prophase (Fig. 2A-b), EGR-1 remains at the centrosomes and the forming mitotic spindle. EGR-1 distributes along the mitotic spindle and the centrosomes in metaphase (Fig. 2A-c). During cytokinesis (Fig. 2A-d), EGR-1 remains at the centrosomes and microtubules and also appears to concentrate at the midbody (open arrow). Centrosomal localization was confirmed by double immunofluorescence with γ-tubulin. No fluorescence was detected in control experiments using the preadsorbed EGR-1 antibody.

Given the dynamic changes observed in the localization of EGR-1 during progression through the cell cycle in BPH-1 cells, we used fluorescence-activated cell sorting (FACS) to determine if EGR-1 is a cell cycle-regulated protein. The FACS profile shown in Fig. 2B demonstrates that the basal levels of fluorescence observed with the control preadsorbed antibody (Fig. 2B-c) increase at least 40 times in the presence of the EGR-1 antibody (Fig. 2B-d). However, these levels do not change through the phases of the cell cycle, demonstrating that EGR-1 expression in BPH-1 cells remains constant throughout the cell cycle. Similar results were obtained with FACS analysis of PC-3 and DU-145 cells, indicating that EGR-1 is not a cell cycle-regulated protein (data not shown). Confocal analysis of flow-sorted BPH-1 cells confirmed that EGR-1 redistributes according to the stage of the cell cycle and that in G2-M (Fig. 2B-d, circle), EGR-1 is associated with the mitotic spindle (Fig. 2C). These data suggest that although the level of EGR-1 expression remains constant in rapidly growing unstimulated BPH-1 cells, the pattern of EGR-1 localization may be regulated according to the distribution of microtubules throughout the cell cycle.

Next, we examined the cellular distribution of EGR-1 in the prostate cancer cell lines PC-3, LNCaP, and DU-145. EGR-1 is localized mainly in perinuclear regions in the PC-3 cells (Fig. 3A-a) as well as in DU-145 cells (Fig. 3, A-c and B). EGR-1 is also associated with centrosomes in these cells (Fig. 5, arrowheads). To determine the subcellular localization of the bright perinuclear EGR-1 immunostaining, cells were analyzed by confocal microscopy after staining with antibodies against microtubules, cytokeratins, Golgi apparatus, and endoplasmic reticulum. Although the filamentous perinuclear expression of EGR-1 in DU-145 cells shows a small overlap with microtubules (Fig. 3A-c), it fully overlaps with cytokeratins (shown by the yellow staining in the overlay image; Fig. 3B), indicating that EGR-1 in these cells is associated primarily with the intermediate filaments. No colocalization was observed with any of the other cellular markers used. The lack of colocalization of EGR-1 and microtubules in PC-3 cells is shown in Fig. 3A-a. In LNCaP cells, EGR-1 appears to have a diffuse cytoplasmic distribution (Fig. 3A-b). Taken together, these results indicate that EGR-1 protein is mainly cytoplasmic in the cancer cells, as observed in the prostate cancer tissue specimens. In addition, EGR-1 appears to be unique among the members of the early growth response family because none of the other related proteins including EGR-2, EGR-3, SP-1, or WT-1 exhibit a pattern of expression similar to that of EGR-1 and most show the expected nuclear staining. Representative examples for EGR-3 and SP-1 are shown in Fig. 3, C and D.

Localization of EGR-1 in the Cytoskeleton of Prostate Cells

The results described above suggest that EGR-1 interacts with the cytoskeleton of prostate cells. To determine whether EGR-1 interacts directly with cytoskeletal elements, the benign prostate cells BPH-1 and RWPE-1 and the prostate cancer cells PC-3, LNCaP, and DU-145 were subjected to a brief (2 min) extraction with a low concentration of nonionic detergent prior to fixation. This pretreatment preserves the cytoskeleton and the proteins interacting with the cytoskeleton but extracts soluble cytoplasmic proteins. After extraction with Triton X-100, cells were immunolabeled with antibodies against EGR-1 and α-tubulin. The association of EGR-1 with microtubules in BPH-1 cells is especially evident in detergent-extracted cells (Fig. 4A-a). In addition, detergent extraction in BPH-1 cells appears to promote a stress stimulus because nuclear localization of EGR-1 was detected in 91.7 ± 2.4% of
the cells compared with nonextracted cells where nuclear localization is observed only in 1.2 ± 1.4% of the cells. This nuclear relocation occurs within the 3–4-min interval between detergent extraction and fixation, suggesting that the nuclear import of EGR-1 in BPH-1 cells is very rapid and may depend on its association with microtubules. Similarly, EGR-1 was associated with microtubules and centrosomes in the benign cell line RWPE-1 with strong perinuclear and a modest, but detectable, nuclear localization. EGR-1 is also associated with centrosomes in these cells (Fig. 4A-b, arrowhead). In the malignant cell line PC-3, cytoplasmic EGR-1 was found surrounding the nucleus, and detergent extraction did not change its localization (Fig. 4A-c). In addition, the use of nonionic detergent did not affect the localization of EGR-1 in DU-145 cells, and as seen before, there was minimum overlap with microtubules (Fig. 4A-d). Nevertheless, in DU-145 cells, EGR-1 was retained on detergent-resistant structures, which had a marked filamentous appearance and are likely to be...
intermediate filaments. It should be noted that in 16 ± 3.3% of DU-145 cells, detergent extraction caused a very modest nuclear relocation of EGR-1. We were unable to characterize the effects of detergent extraction in LNCaP cells because these cells detach from the substrate following detergent treatment. These studies suggest that localization of EGR-1 in the cytoskeleton of BPH-1, RWPE-1, and DU-145 cells and in the nuclear periphery of RWPE-1 and PC-3 cells is stable and that physical methods do not alter this localization, unless they act as a stimulus, as seen in BPH-1 and RWPE-1 cells. The two benign cell lines show a clear microtubule association and nuclear localization of EGR-1, suggesting that nuclear translocation of EGR-1 maybe linked to its localization with microtubules.

To determine whether EGR-1 interacts with microtubule components, EGR-1 was coimmunoprecipitated with anti-tubulin antibodies in BPH-1 cellular extracts in the presence or absence of salt and detergent. As shown in Fig. 4B, γ-, β-, and α-tubulin are present in a protein complex with EGR-1, but this interaction can be easily disrupted with salt and detergent. Nevertheless, considering that EGR-1 interacts with microtubules, we examined the possibility that EGR-1 could be a microtubule-associated protein (MAP). We carried out tubulin polymerization assays in vitro (Fig. 4C) and selective extraction of MAPs (Fig. 4D). In addition to colocalization with microtubules, MAPs follow defined biochemical criteria for their identification. By definition, a MAP is a nontubulin protein that sediments under defined centrifugation conditions and warm temperature, and while in the presence of taxol, it sediments in a taxol-dependent manner (21). The selective extraction approach of Solomon (22) using nonionic detergent in microtubule-stabilizing buffer identifies MAPs as proteins that remain behind in detergent-extracted cytoskeletons of cultured cells and are extracted when cells are pretreated with drugs that depolymerize microtubules. Successive cycles of

![FIGURE 2](image-url). Distribution of EGR-1 in prostate benign BPH-1 cells during the cell cycle. A. Control untreated BPH-1 cells were immunostained simultaneously with anti-α-tubulin (green) and anti-EGR-1 (red) antibodies in combination with Hoechst dye to stain the DNA (blue). Confocal laser scanning microscopy images of α-tubulin (left) and EGR-1 (center) are shown separately for comparison and as an overlay (right). Data show a representative progression through the cell cycle from cells in interphase (a) to mitosis [prophase (b) and metaphase (c)] and cytokinesis (d). These images represent projections of consecutive 0.5-μm optical sections. Arrows, microtubules; arrowheads, centrosomes; open arrow, midbody. The fraction of cells with nuclear localization under these conditions is 3.05 ± 2.8%. B. FACS analysis of EGR-1 protein expression. BPH-1 cells were double stained for DNA with propidium iodide for sorting analysis (a and b) and for EGR-1 using a fluorescein-conjugated anti-rabbit antibody (d). The preadsorbed antibody was used as a control baseline (c). The histograms use arbitrary fluorescence units: green fluorescence for EGR-1 staining is depicted on the Y axis and red fluorescence for DNA content is depicted on the X axis. C. Confocal analysis of flow-sorted BPH-1 cells. A selected cell from the G2-M population (B-d, circle) shows EGR-1 located at the mitotic spindle.
microtubule polymerization and depolymerization in BPH-1 cells demonstrated that EGR-1 remained associated with the cold soluble fraction after the third cycle (Fig. 4C). In addition, in BPH-1 cells treated for 15 min with nonionic detergent in microtubule-stabilizing buffer, EGR-1 was extracted as a soluble protein (Fig. 4D, control 1). Treatment with nonionic detergent and nocodazole, a potent synthetic drug used to depolymerize microtubules (23), also resulted in the complete extraction of EGR-1 in the soluble fraction (Fig. 4D, nocodazole 1). As expected, α-tubulin was present in the soluble fraction and in the pellet of the control cells but only in the soluble fraction of the nocodazole-treated cells. Because it has been reported that p53 associates with microtubules (24), we compared this association under the same rigorous conditions we used for EGR-1. Figure 4D shows that p53 did not cosediment with tubulin in the pellet of the control cells. Thus, based on the criteria of these methods defining MAPs, both approaches suggested that, while EGR-1 and p53 colocalize with microtubules, the interaction is too weak for either protein to be considered structurally a bona fide MAP.

Nuclear Translocation of EGR-1 Requires Intact Microtubules in BPH-1 Cells

The findings above raised an important question about the role of microtubules in the nuclear translocation of EGR-1. To test whether microtubules participate in the nuclear translocation of EGR-1, we used drugs that interact with
microtubules and affect their dynamic behavior by either disassembling them (nocodazole; 23) or stabilizing them (taxol; 25). Rapidly growing BPH-1 cells were serum starved for 18 h and subsequently incubated in the presence or absence of nocodazole (2.5 μg/ml). In preliminary experiments, it was observed that nocodazole alone was a stress stimulus that resulted in a rapid (<10 min) nuclear relocation of EGR-1 in 82.4 ± 2.8% of the cells. Under these conditions, not all microtubules were depolymerized, and we could not define clearly the influence of microtubules in the nuclear translocation of EGR-1. Therefore, a 6-h pretreatment with nocodazole was chosen to clear the stimulation of EGR-1 translocation caused by nocodazole. Nocodazole induced the complete depolymerization of microtubules in BPH-1 cells (Fig. 6A, right). Stimulation of BPH-1 cells with 10% serum after 24 h of serum deprivation resulted in the rapid relocation of EGR-1 into the nucleus of BPH-1 cells (Fig. 6, A, left and D). EGR-1 was detectable inside the nucleus in as little as 15 min and peaked between 30 and 60 min (Fig. 6, A-b, A-c, and D) and returned to basal levels by 3 h (Fig. 6A-d). In contrast, this nuclear translocation of EGR-1 induced by serum was not observed when nocodazole was kept in the incubation media and microtubules remained depolymerized (Fig. 6, A-b', A-c', and D). However, if BPH-1 cells were transferred to media containing 10% serum without nocodazole, EGR-1 colocalized with the nascent microtubules within 10 min (Fig. 6B-a, arrow), and a striking nuclear relocation was evident within 30 min in 96.9 ± 2.5% of the cells (Fig. 6B-b). It should be noted that in some cells, 10 min was sufficient to cause the nuclear translocation of EGR-1. When nocodazole was taken out of the culture media, the repolymerization of microtubules took place within minutes, indicating that the effect of nocodazole was completely reversible and demonstrating that the lack of nuclear translocation of EGR-1 seen in the presence of the drug was not due to toxicity. As shown in Fig. 6, B-a and B-b, EGR-1 localized at the centrosome (arrowhead) and tubulin

**FIGURE 4.** EGR-1 is retained intracellularly after detergent extraction. A. Fixed, detergent-extracted BPH-1 (a), RWPE-1 (b), PC-3 (c), and DU-145 (d) cells were immunostained with antibodies against α-tubulin (green) and EGR-1 (red). Overlay images including labeled DNA (blue) are shown separately. Arrowhead, EGR-1 localization in the centrosomes of RWPE-1 cells. B. BPH-1 cellular extracts were immunoprecipitated with γ, β, and α antibodies and separated by electrophoresis. Western blot analysis was performed with EGR-1 antibody, which recognized the apparent ~80-kDa molecular weight of EGR-1 as a strong band in the control unimmunoprecipitated sample and in the immunoprecipitated samples under mild conditions (arrow). No coprecipitations of EGR-1 and microtubule components were found in the presence of 150 mM NaCl and nonionic detergent. Control immunoprecipitations were performed with mouse ascites fluid (MAF). Arrowheads, the large and small chains of immunoglobulins used for immunoprecipitation. C. In vitro tubulin polymerization assay. BPH-1 cells were fractionated and the starting material (SM), microtubule pellet (P), and soluble (S) protein fractions from three polymerization cycles were immunoblotted with anti-EGR-1 and anti-α-tubulin antibodies. D. Selective extraction of MAPs. BPH-1 cells were exposed to nonionic detergent to separate the soluble proteins (control 1) from the polymerized microtubules (control 2). Treatment with nocodazole for 3 h separates all the soluble tubulin (nocodazole 1) from the background peptides (nocodazole 2). Western blot analysis showed that in both control and nocodazole-treated cells, EGR-1 and p53 were found only in the soluble fraction (1).
polymerization paralleled the localization of EGR-1 in microtubules (arrow). This provides direct evidence that colocalization of EGR-1 with microtubules and the centrosome is an initial event that is followed by subsequent nuclear localization.

As shown above, EGR-1 in prostate cancer cells did not respond to mitogenic stimuli. Serum alone did not induce nuclear relocation of EGR-1 in the cancer cell lines at any of the times tested, and the lack of nuclear translocation was not significantly affected by the presence or the absence of nocodazole (Fig. 6C). A representative example for PC-3 cells is shown in Fig. 6, B-c and B-d, in which EGR-1 remained in a perinuclear location at 10 and 30 min after nocodazole was taken out of the culture medium and in the presence of serum.

When we used taxol in combination with serum in BPH-1 cells, we noticed that taxol alone was also a rapid stimulus for EGR-1 activation (Fig. 7B). However, the activation was much less (37 ± 7.9%) when compared with nocodazole alone (82.4 ± 2.8%). After 1 h of treatment, EGR-1 was retained in microtubule bundles with decreased nuclear translocation, even when taxol incubation media was removed.

**FIGURE 5.** Mitogens are capable of inducing nuclear translocation of EGR-1 in prostate benign cells but not in prostate cancer cells. A. RWPE-1 (a), LNCaP (b), PC-3 (c), and DU-145 (d) cells were grown in basal medium. Cells were stimulated for 1 h with either 50 ng/ml EGF [RWPE-1 (a')] or 10% serum [LNCaP (b'), PC-3 (c'), and DU-145 (d')]. Cells were fixed and subjected to immunofluorescence using antibodies against α-tubulin (green) and EGR-1 (red). DNA was stained with Hoechst dye (blue). The α-tubulin, EGR-1, and DNA staining are shown in the overlay images. Left, control cells; right, stimulated cells. Arrowheads, EGR-1 localization in the centrosomes of PC-3 and DU-145 cells. B. Quantification of EGR-1 nuclear translocation after stimulus. Columns, average of five independent fields of greater than 50 cells; bars, SD. Note the nuclear accumulation of EGR-1 only in benign cells.
Taxol induces the formation of microtubule bundles that, during our experimental time course, remained irreversible. In prostate cancer cells PC-3 and DU-145, taxol had no effect in the cellular localization of EGR-1 (Fig. 7A). Thus, these data confirm our previous conclusion that an intact and dynamic microtubule network is important for nuclear translocation of EGR-1 in benign prostate cells and that EGR-1 in prostate cancer cells is not affected significantly by the use of microtubule-interfering agents.
Therefore, we next sought to determine whether the transcriptional activity of EGR-1 in prostate cells was dependent on the microtubule dynamics. Cells were transfected with a luciferase reporter plasmid, which contains tandem repeats of the EGR-1 consensus binding site. The effect of serum, nocodazole, and taxol on EGR-1 transcriptional activity was tested in prostate cell lines. Figure 7C shows a representative histogram for BPH-1, DU-145, and PC-3 cells.

When cells were deprived of serum for 24 h, the transcriptional activity of EGR-1 was not significantly affected. Serum treatment of transfected cells resulted in a 5-fold increase in EGR-1 transcriptional activity in BPH-1 cells and in a 4-fold increase in DU-145 cells (note that the absolute changes in DU-145 cells are only one-fourth of those observed in BPH-1 cells). In contrast, no changes in the transcriptional activity of EGR-1 were observed in PC-3 cells. Treatment with nocodazole resulted in a significant 3-fold increase in BPH-1 and DU-145 cells, but again, no change was observed in PC-3 cells. The values obtained with nocodazole alone were significantly lower than values obtained with serum alone ($P < 0.001$). These data suggest that whereas serum and nocodazole both stimulate EGR-1 expression in BPH-1 as shown by Western analysis (Fig. 7D), the full transcriptional activity of EGR-1 requires intact microtubules. The benign BPH-1 cell line strongly responded to stimuli. This strong response is evident both at the transcriptional and in the total protein levels. Prostate cancer cells, however, maintain high levels of EGR-1 total protein but are not affected by stimuli (Fig. 7D).

Figure 7D shows that serum, nocodazole, and taxol are stimuli of EGR-1 expression in BPH-1 cells. Serum + nocodazole had additive increases in the EGR-1 transcriptional activity (Fig. 7C). While it seems inconsistent to find low levels of EGR-1 protein with these treatments together, enough time elapsed between both stimuli to clear the EGR-1 protein accumulation. However, serum and nocodazole stimulated EGR-1 expression independently and thus the accumulation of luciferase activity. When taxol was used in combination with serum, it reduced the nuclear localization of EGR-1 (and thus, its transcriptional activity), most likely due to the sequestration of EGR-1 seen in taxol-stabilized microtubule bundles (Fig. 7, A–D). Similarly, the borderline decrease in transcriptional activity observed in PC-3 cells (Fig. 7C) might reflect an
inhibition of RNA trafficking by taxol (26) and thus the translation of the luciferase mRNA.

Discussion

EGR-1 participates in multiple cellular events commonly attributed to its transcriptional activity. In this study, we provide evidence that in benign prostate cells, the nuclear translocation of EGR-1, and thus the transcriptional activation of EGR-1-dependent genes, requires dynamic microtubules. We also show that mitogenic stimulation of prostate cancer cells does not further increase the EGR-1 protein levels or induce nuclear translocation. Our findings provide the first insight into the differences in the intracellular distribution of EGR-1 between benign and cancer prostate cells.

EGR-1 shuttles between the nucleus and the cytoplasm in a tightly regulated fashion in benign prostate cells. Here, we have shown that benign cells maintain low levels of EGR-1 under standard growth conditions and that mitogenic stimuli immediately trigger the expression and nuclear translocation of EGR-1. The concomitant increase in transcriptional activation followed by a rapid decline in EGR-1 levels suggests that an active degradation pathway is intensively dynamic in these cells. The rapid regulation of EGR-1-expression seen in benign prostate cells is characteristic of early response transcription factors. We have shown that in benign human prostate tissues, EGR-1 is localized in some but not all nuclei and that these levels are relatively low when compared with the higher expression in cancer cells. We presume that the nuclear localization of EGR-1 seen in some of the benign cells reflects the cellular response to the stimulus/stimuli causing the hyperplasia or increased proliferation because EGR-1 regulates genes involved in cellular proliferation and cell cycle progression such as growth factors (27–29), cyclin D (30), and thymidine kinase (31). It is logical to assume that benign cells maintain levels of EGR-1 only to the extent needed for biological responses. Clearly, the benign secretory epithelial cells of the nonhyperplastic glands do not maintain detectable EGR-1 expression, and only the basal cells maintain a strong expression. While the role of the basal cells in the prostate is not fully understood, it represents the proliferative compartment of the normal and hyperplastic epithelium (32).

Interestingly, the expression of transforming growth factor-β1, a target for EGR-1 transcriptional regulation that has been implicated in enhancing tumor progression (33), is also seen in the basal cells, in the secretory cells in BPH, and in all epithelial cells in prostate cancer tissues (34). This pattern of transforming growth factor-β1 expression mimics the distribution of EGR-1 that we have observed in the benign and cancerous prostate, suggesting that expression of EGR-1 correlates directly with expression of this target gene in vivo. These observations have been corroborated by Svaren et al. (29) who noted high levels of specific EGR-1 target genes in prostate cancer cells overexpressing EGR-1. However, not all EGR-1 target genes are up-regulated in prostate cancer tissues. In fact, relevant targets such as p53, Rb, the preapoptotic protein Bax, and the corepressor of EGR-1, NAB-2, are down-regulated in prostate cancer tissues (35, 36). While these data may indicate that EGR-1 lacks a transactivation function in prostate cancer, as previously suggested by Ahmed et al. (35), it may also indicate that the target genes activated by EGR-1 in prostate cancer tissues are entirely directed to the maintenance of the malignant phenotype rather than functions that antagonize tumor formation.

NAB-2 is an inhibitor of EGR-1 transcriptional activity (37); therefore, lower levels of NAB-2 allow increased transcriptional activity of EGR-1. Previous observations (36) that NAB-2 levels are diminished in prostate cancer cells relative to benign cells have interesting conceptual implications in light of data presented here. In this study, we show a lower accumulation of EGR-1 protein in the nucleus of prostate cancer cells compared with the cytoplasm in both tissues and cell lines. The absence or low staining in the benign tissue and the detected nuclear translocation in benign cells confirm the high specificity and sensitivity of detection methods. Although not detectable by immunofluorescence staining, the presence of some nuclear EGR-1 protein in prostate cancer cells cannot be disputed because the transfection data show that transcriptional activity of EGR-1 in PC-3 cells is present and at higher levels than in the benign BPH-1 cells. Mitogenic stimuli do not modify protein levels of EGR-1 in malignant prostate cells. However, a small increase at the mRNA level has been seen following stimulation in prostate cancer cell lines (unpublished data), corroborating the work of other authors (38). This minor mRNA regulation fails to translate into protein regulation. The lack of induction of EGR-1 protein has been also observed in two mammary carcinoma cell lines, T47D and ZR-75-1 (12). Thus, our working hypothesis is that relative nuclear levels do not necessarily correlate with transcriptional activity of EGR-1. Furthermore, this activity is altered not only by the nuclear accessibility but also by the lack of transcriptional inhibitors (such as NAB-2) in prostate cancer cells. Thus, while only a small fraction of the EGR-1 overexpressed in cancer cells may reside in the nucleus, these levels may be sufficient to maintain the transcriptional activation of EGR-1 target genes involved in prostate malignancy. Additionally, we have observed a significant increase in the relative half-life of the EGR-1 protein in PC-3 cells versus BPH-1 cells (unpublished data). Therefore, in prostate cancer cells, the absence of NAB-2 together with a longer-lasting EGR-1 provides maximal transcriptional activity that cannot be further increased with stimulation.

With the exception of prostate and some Wilms’ tumors (39), levels of EGR-1 in most cancers are down-regulated compared with benign tissue. Indeed, EGR-1 has been shown to behave as a tumor suppressor in numerous cell lines from nonprostatic origin. The first evidence of EGR-1 promoting the proliferation of transformed cells [shown by Scharnhorst et al. (39) in baby kidney cells in vitro and in vivo] suggested that EGR-1 levels may be differentially regulated in different cancer types and that EGR-1 action is likely to be different and even opposite in various cancer types. Therefore, the recent evidence that the progression of prostate tumors is delayed in EGR-1−/− mice (17) and our unpublished data that blocking EGR-1 expression stops the proliferation of PC-3 cells suggest a tumor-promoting role for EGR-1 in the prostate. Furthermore, while the presence of higher levels of EGR-1 in prostate cancer cells
compared with benign cells is indisputable, the notion that these levels translate into transcriptional activity of EGR-1 is a matter of speculation. Indeed, the two results may be independent of each other.

The cytoplasmic localization of EGR-1 may reflect a difference in regulation of EGR-1 in benign and malignant prostate cells. While the cellular localization and levels of EGR-1 in benign prostate cells are regulated, the levels and localization of EGR-1 in prostate cancer cells are not regulated. A clear result that emerges from our data is that microtubules play a crucial role in the nuclear translocation of EGR-1. Very little is known regarding mechanisms by which "early-acting" transcription factors function so quickly. In the case of EGR-1, the presence of a stimulus causes the nuclear translocation within minutes. At this early time, we presume that the preexisting cytoplasmic pool of EGR-1 translocates into the nucleus. The centrosome may be acting as the cellular reservoir for the EGR-1 protein and thus expedite its nuclear mobilization. The centrosomal pool of EGR-1 is positioned for a very rapid nuclear translocation of EGR-1 due to its close association with the nucleus. The observations that EGR-1 accumulates on microtubules prior to nuclear translocation and nocodazole abolishes the nuclear translocation of EGR-1 suggest that one route for the fast nuclear accumulation of EGR-1 involves recruitment to microtubules and the centrosome via minus end-directed motor proteins. It is of interest to note that the minus end-directed motor protein dynein is required for transport of the tumor suppressor protein p53 to the nucleus along microtubules (24). Our findings also reveal a strong parallel to the early response gene c-myc, which is also overexpressed in prostate tumors (40) and associates with microtubules in cells from various origin (41, 42). Whether c-myc interacts with microtubules in prostate cells remains to be determined.

While we observed a centrosomal localization in most of the cell lines in this study, EGR-1 translocation into the nucleus was observed only in the benign cells. Therefore, while the localization of EGR-1 at centrosomes is not synonymous with nuclear translocation, the colocalization of EGR-1 with microtubules appears to be a requirement for nuclear translocation. Consequently, microtubule composition may be related to the cellular mobilization of EGR-1. In addition, alterations in microtubule composition and MAPs impede the proper mobilization of EGR-1 into the nucleus on stimulation in prostate cancer cell lines. It is notable that the composition of microtubules is altered in many prostate tumors and prostate cancer cell lines (43). Our own unpublished results have corroborated the existence of cell line specific α- and β-tubulin isotype composition. These results lead us to speculate that the association of EGR-1 with microtubules is dependent on the microtubule composition and/or MAPs, which ultimately dictate the nuclear translocation of EGR-1.

It is intriguing that the pattern of localization differs between benign and malignant prostate cells and even between cancer cells. The colocalization of EGR-1 with intermediate filaments, and to a minor extent with microtubules, in DU-145 cells is of particular interest. The close association between intermediate filaments and microtubules and the molecular cross-talk involving the cytoskeletal components (44) provide a mechanism by which EGR-1 could be transported along the microtubules in these cells. This is supported by our data showing that drugs affecting the integrity and dynamics of microtubules in DU-145 cells had the same impact, although to a much lesser extent, on the serum-induced transcriptional activity for EGR-1 as seen in BPH-1 cells. These effects were not apparent by immunofluorescence or Western blots, suggesting that the modest changes in the transcriptional activity of EGR-1 represent only a minor EGR-1 nuclear translocation.

In summary, our results support the hypothesis that rapid nuclear translocation of EGR-1 and accessibility to its target genes is mediated by the cytoskeleton. Our results suggest for the first time that the cytoskeleton localization may be key in the regulation of the tumor promoter role of EGR-1 in prostate cancer cells. They add new insights into the cellular regulation of EGR-1 in both benign and cancerous prostate cells.

Materials and Methods

Cell Lines and Cell Culture

Prostate cancer PC-3, LNCaP, and DU-145 cells were obtained from the American Type Culture Collection (Rockville, MD). PC-3 and LNCaP cells were grown in RPMI 1640 supplemented with 9% fetal bovine serum (FBS) and DU-145 cells were grown in DMEM supplemented with 10% FBS. Immortalized benign prostate BPH-1 cells were a generous gift from Dr. Simon Hayward (University of California, San Francisco, CA). These cells were maintained in RPMI 1640 with 5% FBS. Immortalized benign prostate RWPE-1 cells were a generous gift from Dr. Mukta Webber (Michigan State University, East Lansing, MI). These cells were maintained in keratinocyte serum-free medium supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml EGF. All cells were kept in a humidified atmosphere of 5% CO2 at 37°C.

Immunohistochemistry

Paraffin-embedded prostate tissue sections (5 μm) from 10 BPH and 12 adenocarcinomas were used. Sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Sections were blocked with methanol/hydrogen peroxide and incubated overnight with 0.66 μg/ml of a rabbit polyclonal EGR-1 antibody (sc-110; Santa Cruz Biotechnology, Santa Cruz, CA). EGR-1 immunocomplexes were detected using a biotinylated-labeled anti-rabbit secondary antibody and streptavidin-peroxidase with the Vector Nova Red substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Tissue sections were counterstained with hematoxylin. For staining specificity, tissue sections were incubated with the preadsorbed antibody. To preadsorb the antibody, the control peptide (sc-110-P; Santa Cruz Biotechnology) was incubated overnight with the EGR-1 antibody in 10-fold excess. The preadsorbed antibody was used as a control in all immunostainings and Western blots.

Immunocytochemistry

Cells were grown on glass coverslips, fixed with methanol (−20°C) for 10 min, and permeabilized with 0.1% Triton
X-100 for 2 min. Cells were blocked with 5% goat serum for 1 h and incubated with antibodies against EGR-1 (4 μg/ml, sc-110; Santa Cruz Biotechnology) and α-tubulin (3.3 μg/ml, T9026; Sigma Chemical Co, St. Louis, MO) for 1 h at room temperature (RT). Pan-cytokeratin antibody (C-2931; Sigma Chemical) was used at 20 μg/ml. SP-1 and EGR-3 antibodies (sc-59G and sc-191; Santa Cruz Biotechnology) were used at 5 μg/ml. After washing, the cells were further incubated for 1 h at RT with Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (1:750; Molecular Probes, Eugene, OR). DNA was stained with Hoechst 33342 (0.5 μg/ml; Molecular Probes). Coverslips were mounted with Prolong (Molecular Probes). Fluorescence was detected by confocal laser microscopy (Zeiss LSM 510, Thornwood, NY). For extraction experiments on living cells, media was quickly removed and the cells were incubated for 2 min with 0.1% Triton X-100-containing buffer (50 mM PIPES, 3 mM EGTA, 1 mM MgSO4, 25 mM KCl) prior to fixation. The preadsorbed antibody was used as a control.

Cell Cycle Analysis
Rapidly growing cells were trypsinized, washed with PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 (pH 7.4)], and fixed in 70% ethanol overnight at 4°C. Cells were permeabilized with 0.1% Triton X-100 and processed for immunofluorescence as above. EGR-1 was detected using the primary antibody in combination with a fluorescein-conjugated anti-rabbit secondary antibody. Cells were treated with 0.2 mg/ml RNaseA and resuspended in 20 μg/ml propidium iodide for DNA staining. Cells were analyzed immediately with FACS (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA). Data analyses were performed with the ModFit LT2 Program.

Cellular Extracts and Western Blots
Soluble and insoluble protein extracts were prepared according to the method of Dignam (45) with minor modifications. All procedures were performed at 4°C. Cells were lysed with 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.1% NP40 + protease inhibitors (10 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 mM phenylmethylsulfonfluoride). Supernatants were used for soluble extracts. Pellets were resuspended with 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.5 mM DTT + protease inhibitors. Extracted proteins (from the pellets) were diluted four times with 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT + protease inhibitors. Proteins were measured by the Bradford (46) method and separated by electrophoresis in 8% SDS polyacrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and detected with the polyclonal antibody against EGR-1 using enhanced chemiluminescence.

Identification of EGR-1 as a MAP
To determine whether EGR-1 was a MAP, we performed in vitro tubulin polymerization assays (21). Cells grown to log phase were lysed in a buffer containing 100 mM PIPES (pH 6.9), 1 mM MgCl2, 1 mM EGTA, 0.5% NP40, 10 μg/ml DNaseI, 20 μg/ml RNaseA + protease inhibitors at 4°C for 10 min. The lysate was centrifuged at 100,000 × g for 60 min at 4°C. After centrifugation, the clear supernatant was used as starting material. GTP (1 mM) and taxol (20 μM) were added to the starting material for microtubule assembly at 37°C for 30 min. The sample was centrifuged at 100,000 × g at 37°C for 30 min. The pellet containing the polymerized microtubules and all the MAPs was resuspended in 100 mM PIPES (pH 6.9), 1 mM MgCl2, 1 mM EGTA + protease inhibitors. Microtubules were depolymerized with 3 mM CaCl2 for 1 h at 4°C. The sample was centrifuged at 50,000 × g at 4°C for 30 min. Microtubules were repolymerized as above with EGTA (5 mM), GTP (1 mM), and taxol (10 μM). After centrifugation, the pellet containing microtubules and MAPs was analyzed by Western blots using antibodies against α-tubulin and EGR-1.

A second method for the identification of MAPs was the selective extraction of cultured cells performed as described by Solomon (22). In brief, cells grown to log phase and rinsed with PBS to remove culture medium were extracted in a buffer containing 100 mM PIPES (pH 6.9), 2 mM glyceral, 5 mM MgCl2, 2 mM EGTA, 0.1% NP40 + protease inhibitors at RT for 15 min. This extraction removes the unpolymerized tubulin and 75% of the cellular proteins; left behind are the cytoskeleton preparations containing microtubules and MAPs (fraction 1). Cytoskeleton preparations were incubated in the extraction buffer (without NP40) + 5 mM CaCl2 to release the microtubule depolymerization products (fraction 2). In parallel, an identical extraction was performed in cells preincubated with 2.5 μM nocodazole for 3 h, which produces cytoskeleton preparations lacking microtubules and MAPs, and the further extraction in the presence of calcium releases only background polypeptides. Western blot analysis of proteins from control and nocodazole-treated cells was used to identify the fraction in which EGR-1 concentrates.

Immunoprecipitations
Coimmunoprecipitations for EGR-1 were performed using 50 μg of cellular extracts and 1 μg of γ, β, or α monoclonal antibodies for 2 h at 4°C in low stringency buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl + protease inhibitors] and high stringency buffer (supplemented with 150 mM NaCl and 1% NP40). Twenty microliters of recombinant protein G agarose beads (Life Technologies, Inc., Carlsbad, CA) were added for 45 min at 4°C. The precipitates were washed four times in the same buffer, resuspended in 20 μl of SDS lysis buffer, and analyzed by Western blotting using the sc-110 EGR-1 antibody. Mouse ascites fluid was used as a control for immunoprecipitations.

Cell Transfections and Treatments
Cells were plated in 35-mm dishes to reach 70–80% confluency at the time of transfection. Firefly luciferase reporter plasmids containing EGR-1 binding sites and the empty vector were generously provided by Dr. John Svaren (University of Wisconsin, Madison, WI). All plasmid DNA for transfections was isolated using an endotoxin-free kit (Quagen, Inc., Valencia, CA). Transient transfections were performed using the lipid transfection method.
reagent Gene Porter (Gene Therapy Systems, Inc., San Diego, CA) as per the manufacturer’s instructions. Briefly, 1 μg of the luciferase reporter plasmid containing EGR-1 binding sites was cotransfected with 0.02 μg of Renilla TK luciferase (Promega, Madison, WI) as internal control. Transfections were carried out for 5–6 h in serum-free RPMI 1640 prior to being replaced with the respective complete culture medium (described in “Cell Lines and Cell Culture”) or with RPMI 1640 supplemented with 0.1% FBS. At 12–14 h after transfection, cells were pretreated with nocodazole (2.5 μg/ml) or taxol alone (10 μg/ml) for 2 h prior to stimulation for 6 h with 10% FBS in the presence or absence of nocodazole and taxol. Six hours was the minimum time required to measure the accumulation of luciferase. Control cells were kept in regular media until harvesting. Cell extracts were prepared and assayed for luciferase activity using the dual luciferase reporter system (Promega). Light emission was quantified in a Turner TD-20e luminometer (Sunnyvale, CA). Transfection results were computed as the activity of firefly luciferase relative to the Renilla luciferase. The luciferase reporter without EGR-1 binding sites gave no significant activities.

Statistics
All experiments were repeated at a minimum of three times and representative data were reported. Transient transfection assays were performed in triplicate and repeated four times. Data are presented as means ± SD of triplicates for a representative experiment. Mean ± SD values were analyzed by standard statistical tests for error. Data were analyzed by one-way analysis of variance with post hoc comparison by Tukey’s test. Statistical analysis was performed in triplicate and repeated four times. Data were analyzed by standard statistical tests for error. Data were analyzed by one-way analysis of variance with post hoc comparison by Tukey’s test. Nuclear staining was quantified by analyzing five

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