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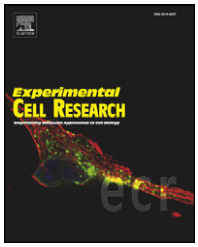

Research Article

The Raf/MEK/extracellular signal-regulated kinase 1/2 pathway can mediate growth inhibitory and differentiation signaling *via* androgen receptor downregulation in prostate cancer cells

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ABSTRACT

Upregulated ERK1/2 activity is correlated with androgen receptor (AR) downregulation in certain prostate cancer (PCa) that exhibits androgen deprivation-induced neuroendocrine differentiation, but its functional relevance requires elucidation. We found that sustained ERK1/2 activation using active Raf or MEK1/2 mutants is sufficient to induce AR downregulation at mRNA and protein levels in LNCaP. Downregulation of AR protein, but not mRNA, was blocked by proteasome inhibitors, MG132 and bortezomib, indicating that the pathway regulation is mediated at multiple points. Ectopic expression of a constitutively active AR inhibited Raf/MEK/ERK-mediated regulation of the differentiation markers, neuron-specific enolase and neutral endopeptidase, and the cyclin-dependent kinase inhibitors, $p16^{INK4A}$ and $p21^{CIP1}$, but not Rb phosphorylation and E2F1 expression, indicating that AR has a specific role in the pathway-mediated differentiation and growth inhibitory signaling. However, despite the sufficient role of Raf/MEK/ERK, its inhibition using U0126 or ERK1/2 knockdown could not block androgen deprivation-induced AR downregulation in an LNCaP neuroendocrine differentiation model, suggesting that additional signaling pathways are involved in the regulation. We additionally report that sustained Raf/MEK/ERK activity can downregulate full length as well as hormone binding domain-deficient AR isoforms in androgen-refractory C4-2 and CWR22R*v1*, but not in LAPC4 and MDA-PCa-2b. Our study demonstrates a novel role of the Raf/MEK/ERK pathway in regulating AR expression in certain PCa types and provides an insight into PCa responses to its aberrant activation.

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Abbreviations: AR, androgen receptor; c.s.FBS, charcoal/dextran-stripped fetal bovine serum; ERK, extracellular signal-regulated kinase; MEK, mitogen activated protein kinase kinase; NEP, neutral endopeptidase; NSE, neuron specific enolase; PCa, prostate cancer; PSA, prostate specific antigen; RSK, ribosomal S6 kinase.

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Introduction

The Raf/MEK/extracellular signal-regulated kinase (ERK) pathway has pivotal roles in controlling cell survival, cell cycle progression and differentiation, and its dysregulated signaling is a central signature of many epithelial cancers [1,2]. The Ser/Thr kinase Raf (c-Raf-1, Raf-B or Raf-A) activates the dual-specificity kinases MEK1 and MEK2 which, in turn, sequentially phosphorylate Tyr and Thr in the activation loop of the ubiquitously expressed Ser/Thr kinases ERK1 and its homologue ERK2. Activated ERK1/2 mediates diverse biological processes by activating/inactivating a wide variety of proteins, and different magnitudes of its activity can lead to distinct biological outputs [1]. Upregulated activity of the MEK/ERK pathway has been shown for its association with progression and poor prognosis of prostate cancer (PCa). For example, ERK1/2 phosphorylation is often detected in correlation with increased tumor grade of primary or metastatic PCa and tumor relapse after therapy [3–6]. In addition, although not frequent, mutations or chromosomal rearrangements of Raf genes that can confer neoplastic Raf kinase activity are detected in human PCa [7–11]. Together, these observations support pathogenetic relevance of the MEK/ERK pathway in prostate tumorigenesis and underscore the need for better understanding of the role of the pathway in PCa cells.

Androgen receptor (AR) is a member of the nuclear receptor superfamily that functions as a ligand-dependent nuclear transcription factor [12]. AR can control the growth regulatory and differentiation pathways in prostate epithelial cells, and its altered expression or signaling is a pivotal event in the carcinogenesis of prostate epithelium [13,14]. While increased AR expression is a key feature of a subpopulation of castration-resistant PCa [15], loss of AR expression, accompanied by decreased mitotic activity, is also detected in certain PCa types that exhibit neuroendocrine (NE) differentiation [16,17]. NE-differentiated cells in PCa are supposed to contribute to castration resistance of neighboring non-NE PCa cells by activating paracrine signaling mechanisms [18,19]. Although the origin of NE differentiated cells in PCa is not fully understood, literatures support the possibility that prostate adenocarcinoma cells transdifferentiate into NE-like phenotype in response to androgen depletion. For example, NE differentiated cells in tumor lesions show identical genetic profiles with adjacent exocrine PCa cells [20], and certain human PCa cell lines, including LNCaP, CWR22 and PC310, exhibit expression of various makers for NE differentiation *in vitro* as well as *in vivo* in response to hormone withdrawal [21–23]. It has been shown that AR downregulation is required for NE differentiation [24], but the signaling mechanisms that underlie AR downregulation upon androgen withdrawal are as yet unclear.

Upregulated ERK1/2 activity is detected in NE cells in PCa as well as in NE-differentiating PCa cell lines while various stimulations that activate the Raf/MEK/ERK pathway can induce NE differentiation of PCa cells in *in vitro* and *in vivo* environments [19,25]. Furthermore, ectopic expression of a constitutively active MEK1 mutant was indeed sufficient to mediate expression of NE markers in LNCaP cells [21]. We recently reported that sustained Raf/MEK/ERK activation can induce cell cycle arrest in G0/G1 phase in LNCaP [26], which may implicate the pathway in decreased mitotic activity of NE-transdifferentiating PCa cells. Together, these results indicate an involvement of Raf/MEK/ERK signaling in NE differentiation of PCa cells. Given the pivotal role of AR for PCa cell proliferation/survival and differentiation, it is conceivable that the Raf/MEK/ERK pathway may also have a role in mediating AR downregulation. Nevertheless, this possibility has never been addressed before.

In this study, we investigated whether the Raf/MEK/ERK pathway can mediate AR downregulation and, thereby, decrease cell proliferation during NE differentiation of LNCaP. We demonstrate that ERK1/2 activity and AR expression are inverse-correlated in an LNCaP model for hormone depletion-mediated NE differentiation and that sustained Raf/MEK/ERK activity is sufficient to mediate AR downregulation, although not necessary. We then examine how AR downregulation is mediated and whether it is required for the pathway-mediated growth arrest and differentiation signaling. Additionally, we show that the pathway can mediate AR downregulation in a subset of PCa lines regardless of androgen sensitivity. This study reveals previously unknown roles of the Raf/MEK/ERK pathway in PCa cells.

Materials and methods

Cell culture, generation of stable line

LNCaP (ATCC), PC3 (ATCC), Du145 (ATCC), and CWR22R*v1* (ATCC) were maintained in phenol red-deficient RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin and 100 μg of streptomycin per ml. LAPC-4 (ATCC) was grown in Iscove's medium with 10% FBS. MDA-Pca-2b (ATCC) was grown in Hams F12 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 25 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, 0.005 mM phosphoethanolamine, 100 pg/ml hydrocortisone, 45 nM selenious acid, 0.005 mg/ml insulin, and 20% FBS. C4-2, NE1.3 and NE1.8 cells were maintained in phenol red-deficient RPMI 1640 supplemented with 10% charcoal/dextran-stripped FBS (c.s.FBS). LNCaPRaf, stably transduced with lentivirus containing ΔRaf-1:ER, was previously described [26]. U0126, MG-132, Lactacystin, and 4-hydroxytamoxifen were obtained from Sigma (St. Louis, MO). Bortezomib was obtained from Selleck Chemicals (Houston, TX).

Viral infection

The lentiviral expression vector pHAGE and the lentiviral shRNA expression vector pLL3.7 (ATCC) were used as previously described [26]. Briefly, for viral production, pHAGE or pLL3.7 was co-transfected with packaging vectors into 293T cells and the resulting supernatant was collected after 48 h. Viral titers were determined by infecting the recipient cell lines with serially diluted viral supernatants mixed with polybrene (Sigma) at 8 μg/ml and scoring cells expressing GFP at 48 h post-infection. Cells were infected overnight and were switched into fresh culture media.

Recombinant lentiviral constructs and RNA interference

Generation of pHAGE-GFP-MEK1CA and pHAGE-GFP-MEK2CA containing constitutively active MEK1-R4F (ΔN3/S218E/S222D) and MEK2-KW71 (ΔN4/S222D/S226D), respectively, were previously described [26]. pHAGE-GFP-H-Ras^{G12V} and pHAGE-GFP-B-