RhoA–ROCK and p38MAPK-MSK1 mediate vitamin D effects on gene expression, phenotype, and Wnt pathway in colon cancer cells

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© 2008 Ordóñez-Morán et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike 3.0 Unported license, and is followed by activation of the p38 mitogen-activated protein kinase (p38MAPK) and mitogen- and stress-activated kinase 1 (MSK1). As shown by the use of chemical inhibitors, dominant-negative mutants and small interfering RNA, RhoA–ROCK, and p38MAPK-MSK1 activation is necessary for the induction of CDH1/E-cadherin, CYP24, and other genes and of an adhesive phenotype by 1,25(OH)2D3. RhoA–ROCK and MSK1 are also required for the inhibition of Wnt-β-catenin pathway and cell proliferation. Thus, the action of 1,25(OH)2D3 on colon carcinoma cells depends on the dual action of VDR as a transcription factor and a nongenomic activator of RhoA–ROCK and p38MAPK-MSK1.

Introduction

The active vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) is a pleiotropic hormone with broad regulatory effects on the proliferation, differentiation, and survival of many cell types (Ordóñez-Morán et al., 2005; Campbell and Adorini, 2006). On the basis of extensive epidemiological and preclinical evidence (Grant and Garland, 2004; Giovannucci et al., 2006; Wu et al., 2007), 1,25(OH)2D3 and several less calcemic derivatives are currently under clinical study alone or in combination as potential agents against colorectal cancer and other neoplasias (Schwartz et al., 2005; Agoston et al., 2006; Deeb et al., 2007). 1,25(OH)2D3 inhibits the proliferation and promotes the differentiation of normal adhesive epithelial phenotype of human colon cancer cells through the transcriptional activation of CDH1 gene encoding E-cadherin and the antagonism of the Wnt–β-catenin signaling pathway (Pálmer et al., 2001), which is aberrantly activated in >80% of human colorectal cancers. E-cadherin is the critical component of the adherens junctions, the intercellular structure needed for the correct formation of compact epithelial layers (Pérez-Morenó et al., 2003). Loss of E-cadherin expression is a requisite for cell deadhesion and migration during the epithelial to mesenchymal
transformation and is common in carcinomas (Takeichi, 1993). Activation of the Wnt–β-catenin pathway by mutation of intracellular components such as APC, AXIN, or CTNNB1/β-catenin genes or epigenetic alteration of Wnt inhibitors such as DKK-1, SFRPs, or WIF is the initial step in colorectal tumorigenesis (van de Wetering et al., 2002; Sancho et al., 2004). The interference of 1,25(OH)₂D₃ with the Wnt–β-catenin pathway relies on the rapid induction of vitamin D receptor (VDR)–β-catenin complexes that titrate out β-catenin, thus hampering formation of the transcriptional competent β-catenin–T cell factor (TCF) complexes that regulate genes involved in tumorigenesis (Pålmer et al., 2001; Shah et al., 2006). Linked to E-cadherin induction, β-catenin later relocates from the nucleus to the adherens junctions (Pålmer et al., 2001). In contrast, the mechanisms leading to the induction of CDH1/E-cadherin and the drastic reorganization of the cytoskeleton by 1,25(OH)₂D₃ remain unknown.

1,25(OH)₂D₃ binds to and activates a member of the superfamily of nuclear receptors, the VDR–β-catenin complexes that titrate out β-catenin, thus hampering formation of the transcriptional competent β-catenin–T cell factor (TCF) complexes that regulate genes involved in tumorigenesis (Pålmer et al., 2001; Shah et al., 2006). Linked to E-cadherin induction, β-catenin later relocates from the nucleus to the adherens junctions (Pålmer et al., 2001). In contrast, the mechanisms leading to the induction of CDH1/E-cadherin and the drastic reorganization of the cytoskeleton by 1,25(OH)₂D₃ remain unknown.

1,25(OH)₂D₃ activates the RhO–ROCK pathway.

Figure 1. 1,25(OH)₂D₃ activates the RhO–ROCK pathway. (A) SW480-ADH cells were treated with 1,25(OH)₂D₃ for the indicated times and RhO activity was determined by GST pulldown. Normalized RhO activity levels are expressed as the mean ± SD (n = 3). (B) 1,25(OH)₂D₃ does not modulate Rac or Cdc42. Levels of active Rac (RacGTP) and Cdc42 (Cdc42GTP) were determined by GST pulldown in cells after 1,25(OH)₂D₃ addition. (C) Scheme of biochemical routes triggered by RhO and sites of inhibition by C3 exoenzyme and Y27632. (D) Cells were treated with 1,25(OH)₂D₃ for the indicated times and the level of phosphocofilin (p-cofilin) and phospho-PRK2 (p-PRK2) were determined by WB. Normalized p-cofilin levels are expressed as the mean ± SD (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

1,25(OH)₂D₃ induces Ca²⁺ influx and activates RhO

As the RhO family of GTPases are key regulators of cytoskeleton dynamics and cell adhesion and migration (Burridge and
imaging, which was sustained as long as 1,25(OH)\textsubscript{2}D\textsubscript{3} was present (Fig. 2 A). Removal of external Ca\textsuperscript{2+} abolished this increase, whereas its readdition induced a small Ca\textsuperscript{2+} overshoot (Fig. 2 B). This finding indicates that the effect was caused the maintenance of Ca\textsuperscript{2+} entry rather than the transient release of Ca\textsuperscript{2+} from intracellular stores. In SW480-R cells, derived from the same parental line as SW480-ADH but expressing very little VDR (Pálmer et al., 2001), 1,25(OH)\textsubscript{2}D\textsubscript{3} induced a much lower, delayed [Ca\textsuperscript{2+}]\textsubscript{cyt} rise that was difficult to distinguish from the gradual increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in vehicle-treated cells (Fig. 2 C). The slight increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in SW480-R cell cultures was caused by only a few cells (Fig. 2 C). Moreover, VDR knockdown by means of small hairpin RNA (shRNA) abolished the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} (see Fig. 8 C). These results indicate that VDR mediates Ca\textsuperscript{2+} influx. However, although the presence of small amounts of VDR in caveolae has been postulated in some cell types (Huhtakangas et al., 2004), we did not detect VDR at the plasma membrane by immunofluorescence. Furthermore, incubation with an anti-VDR antibody did not
RhoA – ROCK activation mediates the induction of phenotypic change and E-cadherin expression by 1,25(OH)₂D₃

To study the role of RhoA activation in 1,25(OH)₂D₃ activity, we first used the C3 exoenzyme transferase, a Rho inhibitor (Fig. 1 C). C3 exoenzyme altered the morphology of untreated cells and blocked the induction of an adhesive phenotype by 1,25(OH)₂D₃ (Fig. 3 A). To confirm the involvement of RhoA in 1,25(OH)₂D₃ activity we generated SW480-ADH cells stably expressing the dominant-negative mutant N19-RhoA (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200803020/DC1). Expression of HA-tagged N19-RhoA did not affect posttranscriptional up-regulation of VDR by its ligand (Wiese et al., 1992; Fig. S2). Likewise, it did not change the predominant nuclear localization of VDR (unpublished data). N19-RhoA cells showed a more rounded, less adherent phenotype than mock-transfected cells (Fig. 3 C) and defects in cytokinesis that lead to polynucleated cells (Glotzer, 2005; Fig. 3 D). The induction of epithelioid islands by 1,25(OH)₂D₃ was impaired in N19-RhoA cell cultures and it was also reduced by treatment with the ROCK inhibitor Y27632 (Fig. 1 C and Fig. 3 C). Immunofluorescence and confocal microscopy showed that 1,25(OH)₂D₃ induced the progressive translocation of RhoA from the cytosol to the cell periphery and its colocalization with actin filaments in SW480-ADH cells (Fig. 3 D). Both expression of N19-RhoA and treatment with
Y27632 inhibited these 1,25(OH)_{2}D_{3} effects (Fig. 3 D). Consistent with the role of the RhoA–ROCK pathway in modulating the actin cytoskeleton (Burridge and Wennerberg, 2004) and with the increase by 1,25(OH)_{2}D_{3} of actin-binding proteins (Pálmer et al., 2003), 1,25(OH)_{2}D_{3} induced (claudin-7) and redistributed (claudin-7 and occludin) tight junction proteins in a RhoA–ROCK–dependent manner (Fig. S2). Y27632 partially disrupted the strong adhesive phenotype of cells stably expressing an exogenous E-cadherin gene (SW480-ADH-E-cadherin) irrespective of 1,25(OH)_{2}D_{3} addition, whereas it did not alter the morphology of E-cadherin and VDR-negative SW480-R cells that do not respond to 1,25(OH)_{2}D_{3} (Fig. 3 B). In contrast, neither the C3 exoenzyme nor Y27632 altered the level of E-cadherin expression in SW480-ADH-E-cadherin cells (Fig. S2). This finding implicates ROCK in E-cadherin–dependent intercellular adhesion. Moreover, Y27632 also altered the adhesive phenotype and impeded 1,25(OH)_{2}D_{3} action in the more differentiated HT29 colon carcinoma cells, in which RhoA was also activated by 1,25(OH)_{2}D_{3} as revealed by the increase in phosducin (unpublished data).

Next we examined whether RhoA–ROCK controls E-cadherin induction by 1,25(OH)_{2}D_{3}. The increase of E-cadherin RNA by 1,25(OH)_{2}D_{3} was reduced by C3 exoenzyme (Fig. 4 A) in N19-RhoA cells (Fig. 4 B) or by addition of Y27632 (Fig. 4 C). The same results were obtained when E-cadherin protein levels were analyzed (Fig. 4, D and E) and on the activation of E-cadherin gene promoter (Fig. 4 F). Collectively, these results indicate that RhoA–ROCK activation is required for the induction of E-cadherin by 1,25(OH)_{2}D_{3}. Accordingly, Y27632 and N19-RhoA inhibited the accumulation of E-cadherin protein at the adherens junctions after 1,25(OH)_{2}D_{3} treatment of SW480-ADH cells (Fig. 4 G).

**RhoA–ROCK activation mediates the gene regulatory action of 1,25(OH)_{2}D_{3}**

We also analyzed the role of RhoA–ROCK in the effects of 1,25(OH)_{2}D_{3} on other targets such as the CYP24 gene. As for E-cadherin, each of the three agents, C3 exoenzyme, N19-RhoA, and Y27632, decreased the activation of the CYP24 gene promoter by 1,25(OH)_{2}D_{3} in SW480-ADH and in SW480-R cells expressing exogenous VDR (Fig. 5, A and B; unpublished data). Accordingly, N19-RhoA and Y27632 also decreased the accumulation of CYP24 RNA (Fig. 5 C). Moreover, the induction of a series of 1,25(OH)_{2}D_{3} targets genes such as osteopontin (OPN), osteocalcin (OCN), and CYP3A at the RNA level and integrin α3, ZO-1, DICKKOPF-1 (DKK-1), p21^{CIP1}, and paxillin at the protein level (Pálmer et al., 2001; Ordóñez-Morán et al., 2005; Aguilera et al., 2007) was blunted in N19-RhoA–expressing cells (Fig. 5, D and E). N19-RhoA also inhibited the induction and accumulation of ZO-1 protein at the membrane where it colocalizes with cortical actin filaments (Fig. 5 F). The same result was obtained using Y27632 (unpublished data). Likewise, N19-RhoA and Y27632 inhibited the increase and redistribution of paxillin and the induction of focal contacts and stress fibers by 1,25(OH)_{2}D_{3} as seen by immunofluorescence (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200803020/DC1). Also in HT29 cells,
RhoA-ROCK activation mediates the antagonism of the Wnt-β-catenin pathway and the inhibition of cell proliferation by 1,25(OH)₂D₃

1,25(OH)₂D₃ induces the relocalization of β-catenin from the nucleus to the plasma membrane adherens junctions, where it binds E-cadherin, thus inhibiting the induction of proliferation and invasion genes by β-catenin–TCF complexes (Pálmér et al., 2001). Coinmunoprecipitation assays revealed that the formation of E-cadherin–β-catenin complexes after 1,25(OH)₂D₃ addition was decreased in N19-RhoA cells as compared with mock cells (Fig. 6 A). Concordantly, the redistribution of β-catenin from the nucleus and cytosol to the plasma membrane induced by 1,25(OH)₂D₃ was partially inhibited in N19-RhoA cells or by Y27632 (Fig. 6 B). Likewise, α- and p120-catenins relocated at the plasma membrane after 1,25(OH)₂D₃ addition and this effect was also diminished in N19-RhoA cells or by Y27632 addition (Fig. 6 B; unpublished data). Moreover, 1,25(OH)₂D₃ did not repress the transcriptional activity of β-catenin–TCF in N19-RhoA cells, whereas it weakly repressed that activity in Y27632-treated mock cells (Fig. 6 C). These results show that RhoA–ROCK activation is required for the antagonism of the Wnt-β-catenin pathway by 1,25(OH)₂D₃. In line with the effect of N19-RhoA in other systems, N19-RhoA cells grew less than mock cells (Fig. 6 D). Additionally, the inhibitory effect of 1,25(OH)₂D₃ on cell proliferation was lost in N19-RhoA cells (Fig. 6 D).
suggesting a role of p38MAPK-MSK1 in mediating 1,25(OH)2D3 action. Furthermore, Ro318220, H89, or SB203580 decreased the phosphorylation of histone H3, an MSK1 substrate (Fig. S4). The link between the activation of p38MAPK and MSK1 was further demonstrated by means of the p38MAPK activator anisomycin, which increased the level of phospho-MSK1 in an SB203580-, H89-, and Ro318220-sensitive manner (Fig. S4).

Consistent with these results, Ro318220, H89, or SB203580, but not GF109203X, Rp-cAMP, or U0126, prevented the induction of E-cadherin RNA (Fig. 7D). Ro318220 also blocked the activation of the E-cadherin gene promoter (Fig. 7E). Moreover, similar results were obtained with CYP24 gene, except that U0126 and Rp-cAMP repressed the accumulation of its RNA (Fig. 7F) and PD98059 repressed the activation of the promoter by 1,25(OH)2D3 (Fig. S4). Furthermore, in HT29 cells the increase of CYP24 RNA expression by 1,25(OH)2D3 was inhibited by Ro318220 or SB203580 (unpublished data). These results implicate p38MAPK-MSK1, MKK1-ERK, and PKA in the induction of CYP24 by 1,25(OH)2D3.

MSK1, PRK2, GSK-3β, and S6K1 (Davies et al., 2000). S6K1 was ruled out, as rapamycin, which specifically inhibits its upstream regulator mTOR (Davies et al., 2000), did not prevent the increase of E-cadherin protein by 1,25(OH)2D3 (Fig. S4). PRK2 was neither activated by 1,25(OH)2D3 (Fig. 1D) nor affected by Ro318220 (Fig. S4). GSK-3β was ruled out as Ro318220 did not increase the level of Snail1 protein, a target of this kinase that accumulates upon GSK-3β blockade by LiCl in SW480-ADH cells (Larriba et al., 2007; unpublished data).

We used additional inhibitors: H89, which inhibits PKA, MSK1, and S6K1 with similar IC50 (Davies et al., 2000), decreased E-cadherin induction; and Rp-cAMP, a specific PKA inhibitor (Davies et al., 2000), had no effect (Fig. 7B). This ruled out PKA and pointed to MSK1. Because MSK1 is activated by either p38MAPK or MKK1–extracellular signal-regulated kinase (ERK; Deak et al., 1998; Dunn et al., 2005), we analyzed the effect of their respective inhibitors, SB203580 and U0126/ PD98059. SB203580, but not U0126 or PD98059, decreased the induction of E-cadherin protein (Fig. 7C; unpublished data), suggesting a role of p38MAPK-MSK1 in mediating 1,25(OH)2D3 action. Furthermore, Ro318220, H89, or SB203580 decreased the phosphorylation of histone H3, an MSK1 substrate (Fig. S4). The link between the activation of p38MAPK and MSK1 was further demonstrated by means of the p38MAPK activator anisomycin, which increased the level of phospho-MSK1 in an SB203580-, H89-, and Ro318220-sensitive manner (Fig. S4).

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We confirmed that 1,25(OH)₂D₃ increased the level of active, phosphorylated p38MAPK and MSK1 without affecting their total cellular content (Fig. 7 G). Furthermore, 1,25(OH)₂D₃ increased phosphorylation of the transcription factors cAMP response element-binding protein (CREB) and activating transcription factor 1 (ATF1), which are MSK1 substrates (see Fig. 9 B). Finally, the finding that knockdown of MSK1 or MSK2 by siRNA decreased the induction of E-cadherin is further evidence that these kinases mediate 1,25(OH)₂D₃ action (Fig. 7 H).

The activation of the signaling pathway by 1,25(OH)₂D₃ requires VDR and takes place in other tumoral and nontumoral cell lines

To examine the dependence on VDR of the different components of the signaling pathway induced by 1,25(OH)₂D₃, we knocked down VDR expression in SW480-ADH cells by means of infection with VDR shRNA lentiviruses. Two VDR shRNA (3 and 4) and one control shRNA cell lines were generated. Both VDR shRNA lines showed less intercellular adhesion and more rounded cells than control shRNA cells in both the absence and presence of 1,25(OH)₂D₃ (Fig. 8 A). We confirmed that VDR shRNA cells expressed less basal and induced VDR protein than control

shRNA cells (Fig. 8 B). In addition, VDR shRNA cells had a lower level of E-cadherin protein and showed no induction of CYP24 promoter in the absence and particularly in the presence of 1,25(OH)₂D₃ (Fig. 8 B). Likewise, the induction of CYP24, OPN, OCN, and CYP3A RNA was blunted in VDR shRNA cells (unpublished data). Remarkably, 1,25(OH)₂D₃ did not increase [Ca²⁺] cyt (Fig. 8 C) and did not activate RhoA (Fig. 8 D), p38MAPK (Fig. 8 E), or MSK1 (Fig. 8 F) in VDR shRNA cells. Consistently, it did not increased CREB phosphorylation (Fig. 8 F). Together, these results show that VDR is needed for the induction of the whole signaling pathway by 1,25(OH)₂D₃.

To know whether p38MAPK-MSK1 mediated the genomic effects of 1,25(OH)₂D₃ in other cell types we used the SB203580 and Ro318220 inhibitors. First, we checked that both agents inhibited efficiently the phosphorylation of CREB in nontumoral human HaCaT keratinocytes and IMR90 fibroblasts, mouse NIH 3T3 fibroblasts, and rat intestine IEC18 cells (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200803020/DC1). Next, the activation of CYP24 promoter by 1,25(OH)₂D₃ was found to be inhibited by Ro318220 or SB203580 in IEC18 and HaCaT and by Ro318220 and H89 in NIH 3T3 cells (Fig. S5). Finally, Ro318220 inhibited the induction by 1,25(OH)₂D₃ of
The activation of p38MAPK-MSK1 is a downstream RhoA-ROCK event that is necessary for the interference of the Wnt-β-catenin pathway by 1,25(OH)2D3. Ro318220 did not prevent the rise in [Ca2+]i, indicating that Ca2+ influx is independent of MSK1 activation (Fig. 9 A). Likewise, Ro318220 neither inhibited the increase in the level of RhoA-GTP (unpublished data) or of phosphorylated cofilin by 1,25(OH)2D3 nor affected phospho-PRK2 (Fig. S4). The activation of p38MAPK and MSK1 by 1,25(OH)2D3 was absent in N19-RhoA cells (Fig. 9 B). In line with this, the level of phosphorylated CREB and ATF1 was increased by 1,25(OH)2D3 in SW480-ADH cells (unpublished data). In addition, SB203580 or Ro318220 further reduced the induction of E-cadherin and β-catenin proteins (left) and CYP24 promoter activity (right) in control and VDR shRNA cells that were treated or not with 1,25(OH)2D3 for 24 h. Protein levels were determined by WB using β-actin as control and CYP24 promoter activity was analyzed as in legend to Fig. 5. [C] Effect of VDR knockdown on the increase in [Ca2+]i induced by 1,25(OH)2D3. The three cell types were loaded with fluo2/AM and assessed for responsiveness to 1,25(OH)2D3. Traces are mean ± SEM values of control cells (black) or the two VDR shRNA lines (red and blue traces). Data are representative of at least two independent experiments for each cell line. (D) Control and VDR-4 shRNA cells were treated with 1,25(OH)2D3 or vehicle for 1 h and RhoA activity was determined by GST pulldown. Normalized RhoA-GTP levels are expressed as the mean ± SD (n = 3). [E] Control and VDR-4 shRNA cells were treated with 1,25(OH)2D3 or vehicle for 2 h and the level of phospho- and total p38MAPK was determined by WB (mean ± SD, n = 3). (F) Control and VDR-4 shRNA cells were treated with 1,25(OH)2D3 or vehicle for 2 h and the level of phospho-MSK1 (p-MSK1) and phospho-CREB (p-CREB) in nuclear (N) and cytosolic (C) fractions was determined by WB. Total CREB and lamin B and β-tubulin were used as respective controls. *, P < 0.05; ***, P < 0.001.

Discussion

Natural and synthetic vitamin D compounds are increasingly studied as anticancer agents (Deeb et al., 2007). Our results highlight a signaling pathway triggered by 1,25(OH)2D3 that starts with the Ca2+ influx from the external medium and continues with the activation of RhoA-ROCK and then of the p38MAPK-MSK1 kinase module. These are necessary steps for the regulation of gene expression, the antagonism of the Wnt-β-catenin pathway, and the induction of an adhesive epithelial phenotype (Fig. 9 F). The Ca2+ influx–RhoA–ROCK–p38MAPK-MSK1 pathway shown here links the nongenomic and genomic 1,25(OH)2D3 effects and demonstrates that the rapid modulation of ion content and cytosolic GTPases and kinases is required for the regulation of gene expression and cell physiology.

The activation of the nongenomic pathway requires VDR as shown by the generation and study of VDR shRNA cells and the analysis of VDR-deficient SW480-R cells. How these rapid and transcription-independent events are triggered is unclear, as we did not detect VDR at the plasma membrane and an anti-VDR antibody failed to block Ca2+ influx by 1,25(OH)2D3. These negative results do not rule out the presence of small amounts of VDR at the plasma membrane, as occurs with estrogen or progesterone nuclear receptors (Losel et al., 2003; Norman et al., 2004). Moreover, the lack of effect on VDR knockdown by means of shRNA and in VDR-deficient SW480-R cells is consistent with the requirement of functional VDR in osteoblasts for the modulation of ion channel responses (Zanello and Norman, 2004).
VDR may not be the only receptor for 1,25(OH)\(_2\)D\(_3\) (Nemere et al., 2004). However, the evidence from studies using cultured cells and genetically modified mice suggests that most 1,25(OH)\(_2\)D\(_3\) actions are mediated by VDR (Erben et al., 2002). We conclude that the initial Ca\(^{2+}\) influx is mediated by cytosolic VDR, although low membrane VDR levels as well as the binding of 1,25(OH)\(_2\)D\(_3\) to a Ca\(^{2+}\) channel or an associated membrane protein cannot be ruled out. The conformational change induced by ligand binding in cytosolic VDR may trigger the initial signal, perhaps indirectly by releasing putative associated factors. The cytosolic fraction of VDR has recently been localized in the vicinity of the plasma membrane in leukemia cells (Gocenk et al., 2007), which would tend to support this hypothesis. Our findings imply a dual action of VDR, as a signaling molecule at the plasma membrane–cytosol and as a transcription factor within the nucleus.

The use of antagonists indicates that L-type voltage-gated calcium channels mediate the Ca\(^{2+}\) influx induced by 1,25(OH)\(_2\)D\(_3\), as it has been recently reported in the case of the vitamin D analogue elocalcitol in human and rat bladder smooth muscle cells (Morelli et al., 2008). Interestingly, the expression of the α1c isoform of this channel is elevated in colon cancer as compared with adjacent normal mucosa (Wang et al., 2000), which is compatible with an effect of 1,25(OH)\(_2\)D\(_3\) in this neoplasia.

The role of RhoA in the induction of CDH1/E-cadherin and other target genes and the profound phenotypic change induced by 1,25(OH)\(_2\)D\(_3\) are consistent with its key function regulating the cytoskeleton, endocytosis, cell polarity, migration, gene transcription, proliferation, differentiation, and oncogenesis (Burridge and Wennerberg, 2004). The inhibitory effects of N19-RhoA and Y27632 on the induction and redistribution of tight junction and adherens junction proteins by 1,25(OH)\(_2\)D\(_3\) indicate that RhoA activation is crucial for the acquisition of polarity and the adhesive phenotype, respectively, which are characteristics of differentiated epithelial cells. Rho GTPases and E-cadherin function control each other: stable localization of E-cadherin at the adherens junctions requires Rho activity, whereas RhoA is inhibited...
ponents of any of the multiprotein complexes (DRIP-TRAP and MSK1 may phosphorylate VDR and/or its coregulators or coactivated with immediate-early gene induction (Thomson et al., 1999). The transient nature of RhoA activation is probably crucial, as we were unable to generate SW480-ADH cells stably expressing constitutively active RhoA caused by cytotoxicity.

MSK1, the downstream kinase activated, is predominantly nuclear although its presence in the cytosol has also been documented, and it participates in the nucleosome response associated with immediate-early gene induction (Thomson et al., 1999). MSK1 may phosphorylate VDR and/or its coregulators or components of any of the multiprotein complexes (DRIP-TRAP and Mediator) involved in transcriptional control by 1,25(OH)2D3-VDR. Perhaps a more plausible role of MSK1 may be the regulation of downstream transcription factors: MSK1 may recruit coactivators or promote interactions with chromatin-modifying or -remodeling complexes through its ability to phosphorylate transcription factors such as CREB or ATF1 or the histone H3 tail (Dunn et al., 2005). Recently, ERK activation by progestins has been reported to cause progesterone receptor phosphorylation and MSK1 activation, which is followed by recruitment of a complex of the three proteins to a nucleosome on the mouse mammary tumor virus promoter and its subsequent induction (Vicent et al., 2006). Putatively, MSK1 (and possibly the 75% homologous MSK2, according to the knockdown experiments) plays similar roles in gene activation by 1,25(OH)2D3. In the case of E-cadherin it depends on p38MAPK-MSK1 activation, but in the case of CYP24 it also depends on MKK1-ERK and PKA. Additionally, the finding that the antagonism of the Wnt-β-catenin pathway by 1,25(OH)2D3, which is mediated by VDR-β-catenin interaction (Palmér et al., 2001; Shah et al., 2006), is sensitive to Ro318220 points to effects of MSK1 at the VDR level.

Our results raise interesting questions such as the role of nuclear versus cytosolic VDR, how Ca2+ can activate RhoA, or which are the MSK1 substrates relevant for 1,25(OH)2D3 action. These questions and whether the Ca2+ influx-RhoA-ROCK-p38MAPK-MSK1 pathway is responsible or for participates in 1,25(OH)2D3 action in other cellular processes will require further work. The identification of this route as crucial for the control of human colon carcinoma cell proliferation and phenotype opens novel possibilities of pharmaceutical intervention for the second most lethal neoplasiaworldwide (Jemal et al., 2006).

Materials and methods

Cell culture and transfections

Tumor-mimetic SW480-ADH and SW480-R cells (derived from the SW480 cell line by limit dilution [Palmér et al., 2001], HT29, Caco-2 (colon) and MCF-7 (breast) cells, and non-tumor human IMR90 (fibroblasts) and HaCaT (keratinocytes), rat IEC18 (intestine), and mouse NIH 3T3 (fibroblasts) cells were cultured in DME plus 10% fetal bovine serum (Invitrogen). SW480-ADH-cadherin cells were previously described (Aguilera et al., 2007). All experiments using 1,25(OH)2D3 [provided by D. Bouillon and A. Verstuyf, Katholieke Universiteit, Leuven, Belgium, and J.P. van de Velde, Solvay, Weep, Netherlands] were performed in DME supplemented with charcoal-treated serum. To ensure dose dependence, 1,25(OH)2D3 was used in the range 10-6 to 10-7 M; data shown correspond to 10-7 M unless specified (see Calcium imaging section). Cells were transfected using the jetPEI reagent (Polyplus Transfection), achieving an efficiency of 60% as judged by fluorescence. In transient transfections, Firefly (Luc) and Renilla reniformis luciferase (RLuc) activities were measured separately using the Dual Luciferase reagent kit (Promega) and a Lumar LB507 luminometer (Berthold). Lucifer activity was normalized to the RLuc activity. The human CYP24 promoter (~367/+1 fragment containing two vitamin D response elements at -293/-273 and -172/-143 positions), luciferase reporter, and VDR expression plasmids were a gift from A. Aranda (Instituto de Investigaciones Biomedicas, Madrid, Spain); and the RhoA constructs were obtained from P. Crespo (Instituto de Biomedicina y Biotecnologia de Cantabria, Santander, Spain). The CDH1/E-cadherin promoter activity was studied using the ~987-TK-Luc construct (Palmér et al., 2001). To study β-catenin–TCF transcriptional activity we used the TOP-Flash and FOP-Flash plasmids containing multimerized wild-type (CCTTGTAC) or mutated (CCTTGGC) TCF/LEF-1 binding sites upstream of a minimal c-fos promoter driving luciferase gene expression (Korinek et al., 1997; a gift from H. Clevers, Hubrecht Institute and University Medical Center, Utrecht, Netherlands). Mock and N19-RhoA cells were generated by stable transfection of SW480-ADH cells with HA-tagged empty vector or N19-RhoA cDNA and selection with 0.3 mg/ml G418 (Sigma-Aldrich).

Gene silencing

To knock down VDR expression, SW480-ADH cells were infected with lentiviral particles containing a U6 promoter driven by an shRNA targeting VDR. These constructs belong to the Mission TRCHs 1.0 shRNA library (Sigma-Aldrich). Infected cells were selected with puromycin at 1 μg/ml. Control cells were infected with lentivirus bearing a nontargeting shRNA that activates the RISC complex and the RNA interference pathway but that contains at least five mismatched nucleotides compared with any human gene (clone SHC002; Sigma-Aldrich). MSK1 and MSK2 siRNA oligonucleotides (siGENOME SMART pool MSK1 and MSK2; Thermo Fisher Scientific) and control oligonucleotides (siControl nontargeting siRNA pool #1; Thermo Fisher Scientific) were transfected using Lipofectamine 2000 in OPTI-MEM medium (both from Invitrogen). Transfection efficiency was judged to be >90% using fluorescence-labeled oligonucleotides.

Inhibitors

The inhibitor C3 exoenzyme (Rho) was obtained from Cytoskeleton, Inc.; Ro318220 (PKC, MSK, p70S6K1, PRK2, OSK-βs, and p90S6K1/RSK1), SB203580 (p38MAPK), GF109203X (PKC), Y27632 (ROCK), H89 (PKA, PKC, MSK1, and S6K), Rp3AMP (PKA specific), PD98059 and U0126 (MKK1), and rapamycin (mTOR-S6K1) were obtained from EMD. The potency and specificity of these kinase inhibitors have been reported elsewhere (Davies et al., 2000). The transcription inhibitors actinomycin D and DRB, the p38MAPK activator anisomycin, and lysophosphatidic acid were obtained from Sigma-Aldrich. Nimodipine and LaCl3 were obtained from Bayer AG and Sigma-Aldrich, respectively.

Antibodies

We used primary mouse monoclonal antibodies against RhoA and phospho-ERK1/2 (Santa Cruz Biotechnology, Inc.); Cdc42, Rac, E-cadherin, β- and p120-catenin, PRK2, and paxillin (BD); integrin α3 (Millipore), β-tubulin (Sigma-Aldrich); p21(CIP1)/waf1 (Millipore), HA (Babco); rat monoclonal antibodies against VDR [Millipore] and MSK2 (R&D Systems); rabbit polyclonal antibodies against RhoA, cyclin D1, and ERK2 (Santa Cruz Biotechnology, Inc.); α-catenin (Sigma-Aldrich); total and phosphohcopholin, phospho-p38MAPK, S6K1, -CREB (Ser133)/ATF1, -MSK1 (Ser581), PRK2, histone H3 (Ser1), and PKD (Cell Signaling Technology); occludin, ZO-1, and claudin7 (Invitrogen); and goat polyclonal antibodies against β-actin, lamin B, MSK1, and DKK1 (Santa Cruz Biotechnology, Inc.).

RNA synthesis

30,000 cells were seeded in 24-well dishes. After overnight incubation, cells were pulsed with 1 μCi/ml [5-3H]uridine 5′-triphosphate (Hartmann Analytical) for 4 h in the presence of the indicated doses of actinomycin D or vehicle (added 30 min before). At the end of the labeling period, the medium was removed and the cells were rinsed twice in PBS and fixed with room temperature for 20 min. Thereafter, precipitated macromolecules were dissolved in 500 μl of 0.5 M NaOH/0.1% SDS and 450 μl of each sample was divided in 5 ml of scintillation solution Optiphase HiSafe 2 (PerkinElmer).
Radioactivity was measured on a 1209 RackBeta counter (LAB Wallac; PerkinElmer).

**Calium imaging**

Cells were plated at ~0.5 × 10^5 cells/ml on 12-mm glass coverslips treated with poly-L-lysine and incubated with 4 μM fura2/AM for 60 min at room temperature in a Hank's medium containing 1.45 M NaCl, 5.65 M KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM Hepes/NaOH, pH 7.4. For Ca²⁺-free conditions, CaCl₂ was replaced with 0.5 mM EGTA. Coverslips were then placed on the heated stage (37 °C) of an inverted microscope (Diaphot [Nikon]; Axiovert S100 TV [Carl Zeiss, Inc.]) and continuously illuminated with a mercury lamp (Orca ER; Hamamatsu Photonics). Image acquisition was done sequentially by direct register using LSM software (LSM 510; Carl Zeiss, Inc.) using plan apochromat immersion oil 63x NA 1.4 objective lens and argon ion (488 nm) and HeNe (543 nm) lasers. Images were acquired sequentially by direct register using LSM software (Carl Zeiss, Inc.) without manipulation; for double labeling experiments, images of the same confocal plane were generated and superimposed. Time-dependent images were acquired with a digital camera (DC300; Leica) mounted on an inverted microscope (Labovert FS; Leitz). Tiff images were processed using Photoshop 7.0 software (Adobe).

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was purified using RNeasy mini kits following the manufacturer’s instructions (QIAGEN). To measure the RNA levels for CDH1, E-cadherin, CYP3A, and CYP24A, we used oligonucleotides (5'-AGAAC-GCATGGCCACAATACCTC-3' and 5'-CATTCTGATCGTGTTGACATG-3') for CDH1/E-cadherin; 5'-TTGCAGTGAATGGCTTGTCG-3' and 5'-GTCGAT-GCTTCGTTGGACG-3' for CYP3A; 5'-AACA-GTCCGAGCAGCTACGC-3' and 5'-CTAGTGCTACCTGCAGCCTG-3' for GAPDH and SBYR green, and for CYP24 and 18S RNA, we used TaqMan probes (Applied Biosystems) with a 7900HT fast real-time PCR system (Applied Biosystems). The PCR cycling conditions used were as follows: incubation at 95 °C for 10 min followed by 40 cycles of 15 s at 93 °C, 60 s at 60 °C, and 5 s at 72 °C. At the end of the PCR cycles, melting curve analyses were performed, and the extent of induction was calculated as described previously (Peralyka et al., 2005).

**Western blotting (WB) and immunoprecipitation assays**

Whole-cell extracts were prepared by washing the monolayers twice in PBS and cell lysis was done by incubation in RIPA buffer (150 mM NaCl, 1.5 mM MgCl₂, 10 mM NaF, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, and 50 mM Hepes, pH 7.4) plus phosphatase and protease-inhibitor mixture (25 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μM PMSF, 1 mg/μl leupeptin, and 10 μg/μl apronitin) for 15 min on ice followed by centrifugation at 13,000 rpm for 10 min at 4°C. To obtain subcellular extracts the cells were washed in cold PBS and disrupted using a hypotonic lysis buffer (0.5% NP-40, 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, pH 7.9, 1 mM DTT, 1 mM PMSF, 1 mM Na₃VO₄, and 10 μg/μl apronitin, leupeptin, and pepstatin). Lysates were centrifuged 11,000 g at 10 min for 4°C, and supernatants containing the cytosolic and membrane protein extract were maintained at ~ 80°C until analysis. Pellets containing nuclei were lysed with hypertonic buffer (0.5% NP-40, 20 mM Heps, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% poly-L-lysine, 1 μM PMSF, 1 mM Na₃VO₄, and 10 μg/μl apronitin, leupeptin, and pepstatin) and centrifuged at 11,000 g at 4°C for 6 min, and supernatants were conserved at ~ 80°C. The protein concentration was measured using the DC protein assay kit (BioRad Laboratories). WB of cell lysates or immunoprecipitates was performed by electrophoresis in SDS gels and by protein transfer to Immobilon P membranes (Millipore). The membranes were incubated with the appropriate primary and secondary horseradish peroxidase-conjugated antibodies, and the antigen binding was visualized using the ECL detection system (GE Healthcare). Quantification was done by densitometry using ImageJ software.

**GST pull-down assays**

For the isolation of the RhoA, Rac, and Cdc42 was assessed by affinity precipitation of the GST-bound form of these GTPases using the GST-hoteken binding domain for RhoA and the GST-CHIB domain of PAK1 for Rac and Cdc42 as described previously (Azim et al., 2000; Ren and Schwartz, 2000b). For in vivo binding assays, human cells were washed twice in ice-cold PBS and lysed, incubated with the GST fusion protein on glutathione-Sepharose 4B beads, and analyzed as described previously (Azim et al., 2000; Ren and Schwartz, 2000b). After the pulldown assay, the eluted active RhoA, Rac, or Cdc42 was detected by WB. Total protein was measured in the cell lysates that were used for the pulldown studies and served as loading controls.

**Immunofluorescence and confocal microscopy**

Cells were rinsed once in PBS, fixed in 3.7% paraformaldehyde for 15 min at room temperature, and rinsed once in 0.1 M glycine and twice in PBS. The cells were permeabilized in 0.5% Triton X-100 and then washed three times in PBS. Nonspecific sites were blocked by incubation with PBS containing 1% goat serum for 30 min at room temperature before incubating the cells with the primary antibodies diluted in PBS for 3 h at room temperature overnight at 4°C. After four washes in PBS, the cells were incubated with secondary antibodies for 45 min at room temperature, washed three times in PBS, and mounted in VectaShield (Vector Laboratories). F-actin was stained with Texas red–labeled phalloidin (Sigma–Aldrich) for 10 min at room temperature followed by four washes in PBS. The secondary antibodies used included the following: FITC-conjugated goat anti–mouse IgG (Jackson Immunoresearch Laboratories) and FITC-conjugated goat anti–rabbit IgG (heavy light chains; Vector Laboratories). Images of immunolabeled samples were obtained at 20°C with a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Inc.) using plan apochromat immersion oil 63× NA 1.4 objective lens and argon ion (488 nm) and HeNe (543 nm) lasers. Images were acquired sequentially by direct register using LSM software (Carl Zeiss, Inc.) without manipulation; for double labeling experiments, images of the same confocal plane were generated and superimposed. Time-course images were captured with a digital camera (DC300; Leica) mounted an inverted microscope (Labovert FS; Leitz). Tiff images were processed using Photoshop 7.0 software (Adobe).

**Online supplemental material**

Fig S1 shows controls for transcription inhibition by actinomycin D and DRB in SW480-ADH cells and the specificity of the increase of [Ca²⁺]cyt by 10⁻⁷ M 1,25(OH)₂D₃ in SW480-ADH cells and of C2 exoenzyme and Y27632 on E-cadherin expression in SW480-ADH-E-cadherin cells and the dependence on RhoA–ROCK of the induction of collagen7 and occludin by 1,25(OH)₂D₃. Fig. S2 shows the lack of effect of N19-RhoA on VDR promoter activation induced by 1,25(OH)₂D₃. Fig. S3 shows the inhibition by N19-RhoA and Y27632 of the induction of [Ca²⁺]cyt by 1,25(OH)₂D₃. Fig. S4 shows a scheme of the action of the kinase inhibitors used, their effect on cell viability, on E-cadherin expression and S6K1 phosphorylation (rapamycin), on histone H3 (GF109203X, Ro318220, SB203580, H89, and Rp-cAMP), on GAPDH and 18S rRNA, we used TaqMan probes (Applied Biosystems) with a 7900HT fast real-time PCR system (Applied Biosystems). The PCR cycling conditions used were as follows: incubation at 95°C for 10 min followed by 40 cycles of 15 s at 93°C, 60 s at 60°C, and 5 s at 72°C. At the end of the PCR cycles, melting curve analyses were performed, and the extent of induction was calculated as described previously (Peralyka et al., 2005).

**References**


