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Membrane androgen receptor characteristics of human ZIP9 (SLC39A) zinc transporter in prostate cancer cells: Androgen-specific activation and involvement of an inhibitory G protein in zinc and MAP kinase signaling

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ABSTRACT

Characteristics of novel human membrane androgen receptor (mAR), ZIP9 (SLC39A9), were investigated in ZIP9-transfected PC-3 cells (PC3-ZIP9). Ligand blot analysis showed plasma membrane [^{3}H]-T binding corresponds to the position of ZIP9 on Western blots which suggests ZIP9 can bind [^{3}H]-T alone, without a protein partner. Progesterone antagonized testosterone actions, blocking increases in zinc, Erk phosphorylation and apoptosis, further evidence that ZIP9 is specifically activated by androgens. Pretreatment with GTPγS and pertussis toxin decreased plasma membrane [^{3}H]-T binding and blocked testosterone-induced increases in Erk phosphorylation and intracellular zinc, indicating ZIP9 is coupled to an inhibitory G protein (Gi) that mediates both MAP kinase and zinc signaling. Testosterone treatment of nuclei and mitochondria which express ZIP9 decreased their zinc contents, suggesting ZIP9 also regulates free zinc through releasing it from these intracellular organelles. The results show ZIP9 is a specific Gi coupled-mAR mediating testosterone-induced MAP kinase and zinc signaling in PC3-ZIP9 cells.

1. Introduction

Androgens regulate a wide variety of reproductive and non-reproductive functions through activation of the nuclear androgen receptor (nAR), an intracellular ligand-inducible transcription factor belonging to the nuclear receptor superfamily (De Gendt and Verhoeven, 2012; Matsumoto et al., 2008). In addition, androgens exert nongenomic steroid actions in numerous cell types through activation of intracellular second messenger pathways (Heinlein and Chang, 2002; Rahman and Christian, 2008; Thomas, 2012). These rapid, nonclassical androgen actions can be modulated in some nAR-positive cells by specific nAR ligands, suggesting they are mediated through the nAR (Gorczynska and Handelsman, 1995; Migliaccio et al., 2000; Zhu et al., 1999). However, nongenomic androgen actions and membrane androgen receptor (mAR) binding have also been reported in cells that are not modulated by nAR-specific ligands, or do not express the nAR (Benten et al., 1999; Braun and Thomas, 2005; Hatzoglou et al., 2005; Konoplya and Popoff, 1992; Sun et al., 2006). Collectively, these studies suggest that many nonclassical, cell surface-initiated androgen actions are mediated by novel mARs unrelated to nARs (Lang et al., 2013), but until recently none of these novel mARs had been identified.

Recently, we discovered a novel mAR, the zinc transporter protein ZIP9 (SLC39A9) (Berg et al., 2014; Thomas et al., 2014b). ZIP9 is one of the fourteen members the ZRT and Irt-like Protein (ZIP) zinc transporter family that have important roles in zinc homeostasis by regulating the transport of zinc across membranes into the cytoplasm from both the extracellular compartment and from intracellular stores (Cousins et al., 2006). ZIP proteins are predicted to have eight transmembrane domains and have been classified into four subfamilies (Fukada and Kambe, 2011). ZIP9 is the shortest member of the ZIP family (307–310 amino acids) and the sole member of the ZIP I subfamily (Gaither and Eide, 2001). The ZIP9 cDNA was initially identified as a mAR in Atlantic croaker ovaries using a similar approach to that used to identify membrane progestin receptor alpha (mPRα/PAQR7) in spotted seatrout ovaries

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(Berg et al., 2014; Zhu et al., 2003). Wild-type and recombinant croaker ZIP9 are expressed on the plasma membranes of croaker ovarian granulosa cells and transfected SKBR3 breast cancer cells, respectively, and display high affinity (K_d 12.7 nM), limited capacity, specific [^{3}H]-T binding characteristic of a mAR (Berg et al., 2014). Testosterone activates a stimulatory G protein, increases intracellular cAMP and free zinc levels, and induces apoptosis in croaker ZIP9-transfected cells. Knockdown of ZIP9 expression in croaker granulosa/theca cells with ZIP9 siRNA decreased specific membrane [^{3}H]-T binding and the testosterone-induced increase in intracellular zinc and apoptosis, whereas treatment with croaker nAR siRNA was ineffective (Berg et al., 2014). Parallel studies on wild-type and recombinant human ZIP9 in breast and prostate cancer cell lines showed that human ZIP9 has similar androgen binding characteristics to that observed with croaker ZIP9 and displays low binding affinity for nAR-selective agonists (Thomas et al., 2014b). Human ZIP9 also has similar intracellular signaling and apoptotic functions to those of croaker ZIP9. Treatment of MDA-MB-468 breast cancer cells and human ZIP9-transfected PC-3 prostate cancer cells (PC3-ZIP9) with testosterone, but not with the nAR agonist mibolerone, caused activation of G proteins and second messenger pathways and increases in intracellular zinc which were associated with increases in pro-apoptotic gene expression and induction of apoptosis (Thomas et al., 2014b). Collectively, these studies provide clear evidence that the zinc transporter protein, ZIP9, functions both as a mAR that signals through G proteins and as an androgen-dependent zinc transporter and is an intermediary in androgen induction of apoptosis and death of diverse types of vertebrate cells. Recently, the role of ZIP9 as a mAR that acts through activation of a G proteins has been confirmed in Sertoli and spermatogenic cells by another research group (Bulldan et al., 2016; Shihan et al., 2016).

While these initial studies provide clear evidence that ZIP9 is a unique steroid receptor that has both mAR and zinc signaling functions, several questions arose from the results that require further investigation. The discovery that ZIP9 functions as a mAR is unprecedented for ZIP proteins so it is necessary to determine whether ZIP9 can bind testosterone alone or whether it is a partner with another protein that contains the ligand binding site. Previously, evidence has been obtained of possible cross-talk of androgens with other receptors, including opioid and nerve growth factor receptors, resulting in modulation of nongenomic androgen actions (Anagnostopoulou et al., 2013; Lang et al., 2013). The class C orphan GPCR, GPRC6A, is a potential ligand binding partner with ZIP9 because it has been shown to bind testosterone and proposed to be a mAR (Pi et al., 2010, Pi and Quarles, 2012). However, GPRC6A interacts with a bewildering array of compounds including other steroids, L-α-amino acids, cations, and osteoclathin (Pi and Quarles, 2012), which suggests that instead it may act as an adaptor protein for a variety of proteins including a mAR. Progesterone membrane receptor component 1 (PGRMC1) has been shown to have this function as an adaptor protein for mPRα and EGFR, interacting with these receptors and facilitating their expression on the cell surface (Ahmed et al., 2010; Aizen and Thomas, 2015; Thomas et al., 2014a). Moreover, the steroid specificity of the human ZIP9 receptor is unresolved. Progesterone was an effective competitor of [^{3}H]-T binding to plasma membranes of MDA-MB-468 and PC3-ZIP9 cells (Thomas et al., 2014b), but the binding results may have been confounded in these cancer cell lines by their abundant expression of mPRs which have relatively high binding affinities for testosterone (Pang and Thomas, 2011; Supplementary Fig. 1).

Although human ZIP9 was shown to activate an inhibitory G protein (G_i) in MDA-MB-468 and PC3-ZIP9 cells (Thomas et al., 2014b), efforts to co-immunoprecipitate ZIP9 and the $G_i\alpha$ subunit were unsuccessful. Consequently, it remains unclear whether ZIP9 is directly coupled to an inhibitory G protein in the plasma membranes of these cancer cells. The current model of zinc transport by ZIP family members does not involve G protein activation (Cousins et al., 2006; Fukada and Kambe, 2011), so it is not known whether G_i protein activation is required for the testosterone-mediated zinc signaling functions of ZIP9. In addition to its localization on the plasma membranes of MDA-MB-468 and PC3-ZIP9 cells, the ZIP9 protein has also been detected on nuclear and mitochondrial membranes (Thomas et al., 2014b). Currently, no information is available on the functions of ZIP9 at these subcellular locations and whether nuclear and mitochondrial ZIP9s act as mARs and transport zinc.

Therefore, in the present study additional receptor and signaling characteristics of human ZIP9 were investigated in PC3-ZIP9 cells. Ligand blot analyses of [^{3}H]-T binding to ZIP9 as well as possible interactions of the receptor with GPRC6A were investigated to determine if the [^{3}H]-T binding can be explained by the presence of ZIP9 protein alone. The specificity of testosterone and progesterone binding to ZIP9 was examined in the absence of confounding results obtained through their interactions with the mPRs by subtracting the specific binding of testosterone and progesterone to PC-3 vector (PC3-vect) cells from that to the PC3-ZIP9 cells. Direct G protein coupling to ZIP9 was examined using an *in situ* ligation proximity assay and its role in [^{3}H]-T binding and MAP kinase and zinc signaling were investigated after receptor-G protein uncoupling or reduced numbers of androgen binding sites in the membrane by treatments with GTPγS and pertussis toxin (Pace and Thomas, 2005). Finally, the mAR [^{3}H]-T binding and zinc signaling functions of ZIP9 in other cellular locations was investigated using isolated nuclei and mitochondrial preparations.

2. Materials and methods

2.1. Chemicals

All steroids and chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. [1,2,6,7-^{3}H]-Testosterone ([^{3}H]-T; 83.4 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). Antibodies against Erk 1/2 and phospho-Erk 1/2 (p42/44), were purchased from Cell Signaling Technologies (Danvers, MA). The MEK1/2 inhibitor PD98059 was acquired from Enzo Life Sciences (Farmingdale, NY).

2.2. ZIP9 and GPRC6A transfection and cell culture

The human ZIP9 cDNA clone (SLC39A9, OriGene, Rockville, MD), and human GPRC6A cDNA clone (OriGene) were sub-cloned into a pCMV6-Neo mammalian expression vector with a Myc-DDK tag (OriGene). The ZIP9 construct or empty vector were transfected into human PC-3 cells and the GPRC6A construct or vector were transfected into HEK293 cells (American Type Culture Collection, Manassas, VA) with Lipofectamine 2000 (Invitrogen, Carlsbad CA) following the manufacturer's instructions. The cells were cultured in DMEM/Ham's F12 nutrient medium containing 10% charcoal-stripped fetal calf serum and transfected cells were selected with 500 μg/ml geneticin (G418, Invitrogen). Transfected cells were used after 2–3 passages when ZIP9 and GPRC6A expression was maximal. MDA-MB-468 and MDA-MB-231 breast cancer cells that had not been transfected with ZIP9 or empty vector were transiently transfected twice at 0 h and 16 h with ZIP9 siRNA and non-target siRNA (100 nM, SmartPool, Dharmacon, Lafayette, CO) using Lipofectamine 2000, and GPRC6A mRNA expression was measured by qRT-PCR 2 days later.

Cells were serum starved overnight prior to experimentation