BBA - Molecular Cell Research 1865 (2018) 354–363

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Selective inhibition reveals cyclin-dependent kinase 2 as another kinase that phosphorylates the androgen receptor at serine 81

Radek Jorda[a,*,1], Zuzana Bučková[a,1], Eva Řezníčková[a], Jan Bouchal[b], Vladimír Kryštof[a]

[a] Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University Olomouc & Institute of Experimental Botany ASCR, Šlechtitelů 27, 78371 Olomouc, Czech Republic

[b] Department of Clinical and Molecular Pathology, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University Olomouc, Hněvotínská 5, 779 00 Olomouc, Czech Republic

ARTICLE INFO

Keywords:
Androgen receptor
Cyclin-dependent kinase
Phosphorylation
Serine 81
Inhibitor

ABSTRACT

Several studies have revealed that cyclin-dependent kinases (CDK) can mediate phosphorylation of steroid receptors at multiple sites, including serine 81 of the androgen receptor (AR). Phosphorylation of S81 is required for AR nuclear translocation, an association with chromatin and also regulates endogenous AR-regulated transcription in response to hormones. Up to date, S81-phosphorylation has been studied using different CDK inhibitors. Nevertheless, most inhibitors are non-selective or have unknown selectivity. We investigated the selectivity of commercially available CDK inhibitors and identified compounds that will be suitable for further studies to identify the CDKs responsible for S81-AR phosphorylation. We confirmed the positive impact of CDK1 and CDK9 on phosphorylation of S81-AR and its transcriptional activity. Although CDK1-mediated phosphorylation was previously shown to occur during mitosis, our experiments did not confirm this finding. By using chemical and genetic inhibition techniques, we identified that CDK2 contributes to S81-AR phosphorylation and transactivation while CDK4 was not shown to be involved in this process.

1. Introduction

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that belong to the CMGC group of kinome and their activities are regulated by cyclin binding and inhibitors from the CIP/KIP and INK4 families in response to several extra- and intracellular signals [1,2]. Deregulation of CDKs is frequently associated with human cancers [3,4], and therefore, the development of synthetic inhibitors for therapeutic purposes has recently resulted in the approval of palbociclib (PD0332991, Ibrance®) and ribociclib (LEE011, Kisqali®) for the treatment of advanced breast cancer [5–7]. The CDK family comprises 20 members that are responsible for the phosphorylation of substrates that are important for cell cycle progression and transcription. Several studies have revealed that CDKs can also mediate steroid receptor phosphorylation at multiple sites, including the androgen receptor [8–10].

The androgen receptor (AR) is a steroid hormone receptor that plays a crucial role in the normal development of male reproductive tissues, and its high expression and/or relaxation of its regulation are strongly implicated in prostate cancer (PCa) [11]. Androgen binding induces conformational changes of AR that influence its interactions with other proteins and DNA as well as its subcellular localization and transcriptional activity. AR is further regulated by numerous post-translational modifications (PTMs) that affect its physiological role, especially its transcriptional program. Most phosphorylation events are mediated by different kinases, including phosphorylation of serine, threonine or tyrosine residues, which are distributed along the whole AR sequence, unlike other types of PTMs [8,10]. While the proximal kinases for some phosphosites have been identified, the function of several phospho-residues and the kinases responsible for their phosphorylation are still unknown. One of the most frequently studied phosphosites, serine 81, has been shown to be phosphorylated by the CDK1, CDK5 and CDK9 kinases [12–16]. Notably, overexpression of CDK1 and cyclin B have been suggested to contribute to increased AR activity in prostate cancer and its resistance to AR antagonists [16]. Several studies have suggested that CDK1 can also cooperate with CDK7 and CDK11 to phosphorylate the other two residues of the androgen receptor, serine 515 [17–19] and serine 308 [20,21], respectively.

Our study focuses on the phosphorylation of S81, the function of

Abbreviations: CDK, cyclin-dependent kinase; PTMs, post-translational modifications; AR, androgen receptor; PCa, prostate cancer; PSA, prostate specific antigen; ENZ, enzalutamide; CSS, charcoal stripped serum; FBS, fetal bovine serum; ER, estrogen receptor; PR, progesterone receptor; DRB, 5,6-dichloro-1-*D*-ribofuranosylbenzimidazole

* Corresponding author.
E-mail address: radek.jorda@upol.cz (R. Jorda).
[1] these authors contributed equally.

https://doi.org/10.1016/j.bbamcr.2017.11.011
Received 30 September 2017; Received in revised form 10 November 2017; Accepted 16 November 2017
Available online 20 November 2017

which was previously investigated using various approaches, including S81-phosphosite mutants, AR overexpression, inhibition, siRNA, chromatin-immunoprecipitation and cell reporters [12–16]. Previous studies have demonstrated that S81 phosphorylation is required for AR nuclear translocation and association with chromatin and also regulates endogenous AR-regulated transcription in response to hormones, resulting in positive effects on cell growth.

Previous reports were mostly supported by chemical inhibition of CDKs, but in our opinion, the observed effects are usually pharmacologically corroborated using non-selective CDK inhibitors or CDK inhibitors with unknown selectivity. The use of inhibitors with unclear selectivity might lead to misleading conclusions, especially when these inhibitors are used as a tool for evaluating certain biological processes that are linked with the function of a particular CDK. Roscovitine is an example, and it was initially believed to be a relatively specific inhibitor of CDK1, 2 and 5. However, subsequent studies have demonstrated that it also blocks transcription through inhibition of CDK7 and 9 [22,23]. Importantly, at least two groups used roscovitine as a tool in studies related to the function of AR and showed its ability to block androgen-stimulated phosphorylation of S81 of AR in LNCaP cells [12,15], but each group pointed to a different CDK as the responsible enzyme. Independently, CGP74514A was shown to explain the role of CDK1 in the phosphorylation of S81-AR [13]. Nevertheless, the available selectivity profile and our own data suggest that this compound is less selective and not suitable as a tool.

We therefore decided to verify the potential role of different CDKs in the phosphorylation of S81-AR using a combination of chemical inhibition and siRNA silencing. We systematically characterized the selectivity of different CDK inhibitors on a panel of recombinant CDK/cyclin complexes to complete or verify information that was already published. To improve our understanding of the selectivity of inhibitors, we evaluated their cellular activities in PCa cells by monitoring their substrates using immunoblotting analysis. A key objective of this part of the study was to identify the optimal concentrations of inhibitors and treatment durations for the inhibition of particular CDKs without side effects (e.g., off-target effects or massive apoptosis). Most selective compounds were then used for analyses of the phosphorylation of androgen receptors at S81. A comparison of transient silencing of individual CDKs in PCa cells correlated well with the effects of pharmacological inhibition of CDKs as well as with the AR transcriptional activity in a cellular reporter.

We confirmed the positive impact of CDK1 and CDK9 on the phosphorylation of S81 of AR and its transcriptional activity. Although CDK1-mediated phosphorylation has been shown to occur during mitosis, our experiments did not confirm this finding. Using chemical and genetic inhibition techniques, we showed that CDK2 can contribute to S81-AR phosphorylation and transactivation, while CDK4 was shown to not be involved in this process. Taken together, our observations contribute to the further understanding of the relationship between S81-AR and CDKs.

2. Materials and methods

2.1. Reagents

Specific reagents were purchased from PerkinElmer (R1881), Sigma Aldrich (nocodazole, RO3306, PHA767491), MedChemExpress (abemaciclib, palbociclib, LDC000067), Santa Cruz Biotechnology (NU6102, GW8510), Enzo Life Sciences (CGP74514A) and Calbiochem (SU9516). Enzalutamide was kindly provided by Dr. Frederic R. Santer from Innsbruck Medical University. Specific siRNAs were purchased from Dharmacon (CDK9, L-003243-00-0020; CDK1, L-003224-00-0020; CDK2, L-003236-00-0020; CDK4, L-003238-00-0010) or Santa Cruz Biotechnology (control siRNA, sc-37007).

2.2. Cell lines and transfection

Prostate cancer cell lines (C4-2, LAPC-4 and LNCaP) were authenticated using the AmpFLSTR® Identifiler® PCR Amplification Kit (Applied Biosystems). LNCaP cells were purchased from ECACC. LAPC-4 and C4-2 cells were kindly provided by Dr. Frederic R. Santer from Innsbruck Medical University (Austria) and Dr. Marián Hajdúch from Palacký University in Olomouc (Czech Republic), respectively. The 22Rv1-ARE14 reporter cell line was a generous gift from Dr. Zdeněk Dvořák from Palacký University in Olomouc (Czech Republic) [47]. The C4-2 and LNCaP cell lines were grown in 25 mM HEPES-modified RPMI-1640 medium, the LAPC-4 cell line was grown in Iscove's Modified Dulbecco's medium, and the 22Rv1-ARE14 cell line was grown in RPMI-1640 medium. All media were supplemented with 10% normal or charcoal-stripped fetal bovine serum, 4 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. Cells were maintained in a humidified CO_2 incubator at 37 °C. The cells were transfected by siRNAs specific to certain CDKs using Lipofectamine® RNAiMAX (ThermoFisher Scientific) according to the manufacturer's protocol.

2.3. Immunoblotting and antibodies

Briefly, treated cells were harvested and then lysed in RIPA buffer. Proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking the membranes, they were incubated with specific primary antibodies overnight, washed and then incubated with peroxidase-conjugated secondary antibodies. Finally, peroxidase activity was detected using Pierce™ ECL Western blotting substrates and a CCD camera LAS-4000 (Fujifilm). The specific primary and secondary antibodies are listed in Supplementary Table S2.

2.4. Kinase inhibition assay

CDK/cyclin complexes were assayed with appropriate substrates in the presence of ATP (see Supplementary Table S3), 0.05 µCi [γ-^{33}P]ATP and the test compound in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 2.5 µg/50 µl $PEG_{20.000}$) to a final total volume of 10 µl. The reactions were stopped by adding 5 µL of 3% aq. H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3 × with 0.5% aq. H_3PO_4 and air-dried. Kinase inhibition was quantified using an FLA-7000 digital image analyser (Fujifilm). The concentration of the test compounds required to decrease CDK activity by 50% was determined from the dose-response curves and designated as IC_{50}.

2.5. Cell cycle analysis

PCa cells were treated with tested compounds at different concentrations for 24 h and were then trypsinized, washed with PBS, fixed with ice-cold 70% ethanol and incubated on ice overnight. Following hydration, the cells were stained with propidium iodide (0.01 mg/ml, Sigma Aldrich) for 1 h at room temperature in the dark and finally analysed by flow cytometry using a 488-nm laser (BD FACS Verse with software BD FACSuite™, version 1.0.6.).

2.6. AR-transcriptional reporter assay

22Rv1-ARE14 cells were seeded into a 96-well plate. The next day, the cultivation medium was discarded and the cells were incubated in the absence or presence of tested compounds dissolved in RPMI-1640 medium supplemented with charcoal-stripped serum and 1 nM R1881 for 7 h. After an incubation period, the cells were washed with PBS and lysed for 10 min in a lysis buffer (10 mM Tris pH = 7.4, 2 mM DCTA, 1% nonidet P40, 2 mM DTT). After lysing, a flash mix solution (20 mM tricine pH = 7.8, 2.67 mM $MgSO_4$, 1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2$,