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Protein and its Function Based on a Subcellular Localization

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ABSTRACT

Protein $p21^{Cip1/Waf1}$ is a cyclin-dependent kinase inhibitor, which is important in the response of cells to genotoxic stress and a major transcriptional target of p53 protein. Based on the localization, $p21^{Cip1/Waf1}$ protein executes various functions in the cell. In the nucleus $p21^{Cip1/Waf1}$ binds to and inhibits the activity of cyclin dependent kinases Cdk1 and Cdk2 and blocks the transition from G1 phase into S phase or from G2 phase into mitosis after DNA damage. This enables the repair of damaged DNA. $p21^{Cip1/Waf1}$ was also found as an important protein for the induction of replication senescence as well as stress-induced premature senescence. In the cytoplasm, $p21^{Cip1/Waf1}$ protein has an anti-apoptotic effect. It is able to bind to and inhibit caspase 3, as well as the apoptotic kinases ASK1 and JNK. The function of $p21^{Cip1/Waf1}$ in response to a DNA damage probably depends on the extent of the damage. In the case of low-level DNA damage, the expression of $p21^{Cip1/Waf1}$ is increased, it induces cell cycle arrest, and performs also anti-apoptotic activities. However, after extensive DNA damage the amount of $p21^{Cip1/Waf1}$ protein is decreased and the cell undergoes apoptosis. Dual function of $p21^{Cip1/Waf1}$ was also observed in cancerogenesis. On the one hand, $p21^{Cip1/Waf1}$ acts as a tumor suppressor; on the other hand it prevents apoptosis and acts as an oncogene. Better understanding of the role of $p21^{Cip1/Waf1}$ in various conditions would help to develop better cancer-treatment strategies. J. Cell. Biochem. 112: 3502–3506, 2011.

KEY WORDS: $p21^{Cip1/Waf1}$; SUBCELLULAR LOCALIZATION; SENESCENCE

From the year 1979, when the p53 protein was found [DeLeo et al., 1979], it has become the most intensively studied subject of research in the area of molecular oncology and it is still considered to be one of the most important regulators of cell response to genotoxic stress [Uldrijan et al., 2002]. The exposition of the cell to genotoxic stress (ionizing radiation, UV radiation, chemical mutagens, and cytostatics) can lead to the formation of various DNA damage. Cells try to eliminate this damage to prevent genomic instability and to avoid cancer formation. Signal cascades resulting in three fundamental processes are activated immediately: the cell cycle arrest, the reparation of damaged structure of DNA, and the apoptosis induction [Cazzalini et al., 2010]. In these cascades the $p21^{Cip1/Waf1}$ protein, the expression of which is controlled by the p53 protein, plays an essential role.

The $p21^{Cip1/Waf1}$ protein was identified as the first inhibitor of cyclin-dependent kinases (CDKs). It effectively inhibits cyclin-dependent kinases Cdk2, Cdk3, Cdk4, and Cdk6, which have a direct function in the transition between cell cycle phases [Gartel and Tyner, 1999]. Due to its ability to bind and interact with Cdk, it was named CIP1 (Cdk interacting protein 1). It was also indentified as the product of a gene activated by wild-type p53, and it was named WAF1 (wild-type p53 activated factor) [Gartel and Tyner, 1999].

Protein $p21^{Cip1/Waf1}$ was discovered in 1993 [El-Deiry et al., 1993; Harper et al., 1993]. It is a polypeptide containing 164 aminoacids, its molecular weight is 20 kDa and it is coded by the gene CDKN1A. After DNA damage $p21^{Cip1/Waf1}$ causes cell cycle arrest in G1 phase [Ju et al., 2006], but it also plays a key role in the arrest in the G2/M transition [Taylor and Stark, 2001; Cazzalini et al., 2010].

The function of the $p21^{Cip1/Waf1}$ protein depends on its localization in the cell; it plays different roles in nucleus and in cytoplasm.

POSTTRANSLATION MODIFICATIONS

For subcellular localization of $p21^{Cip1/Waf1}$ protein, its phosphorylations on serin or threonin residues is very important. $p21^{Cip1/Waf1}$ can be phosphorylated by various kinases—the most important phosphorylations are considered those at Thr57, Thr145, Ser146, and Ser130 (see Fig. 1). The exact roles of particular phosphorylation sites are still being explored. It was found, that these phosphorylations—at Thr57 by MST1 (mammalian Sterile20-like 1); at Thr145 and Ser146 by AKT, or at Ser130 by p38 and JNK (Jun N-terminal kinase)—increase the stability of $p21^{Cip1/Waf1}$ protein [Hwang et al.,

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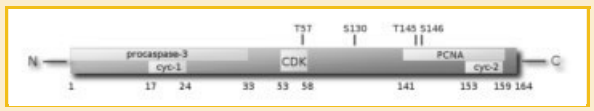

Fig. 1. Binding sites of $p21^{Cip1/Waf1}$ and its most important phosphorylations (cyc-1, cyc-2—binding sites for cyclins, CDK—binding site for cyclin-dependent kinase, PCNA—binding site for proliferating cell nuclear antigen).

2009]. It was proved, that phosphorylation of $p21^{Cip1/Waf1}$ by AKT kinase leads not only to stabilization, but also to distribution of $p21^{Cip1/Waf1}$ into the cytoplasm. There not only degradation occurs: but on the contrary, $p21^{Cip1/Waf1}$ participates in many other processes [Blagosklonny, 2002; Sohn et al., 2006].

On the other hand, phosphorylation catalyzed by extracellular regulated MAP kinase (ERK)—at Thr57 and Ser130, and catalyzed by GSK3 (at Thr57)—was reported to promote $p21^{Cip1/Waf1}$degradation.

ERK is also known to be involved in the stabilization of $p21^{Cip1/Waf1}$, in response to DNA damage it potentiates the anti-apoptotic function of $p21^{Cip1/Waf1}$ and also increases its level in cytoplasm. It was found that the inhibition of ERK cascade in response to DNA damage decreases $p21^{Cip1/Waf1}$ levels in the cytoplasm and $p21^{Cip1/Waf1}$ is increased only in the nucleus. This results show the participation of ERK cascade on the regulation of cytoplasmic localization of $p21^{Cip1/Waf1}$after DNA damage [Heo et al., 2011].

Therefore, it seems that phosphorylation of $p21^{Cip1/Waf1}$protein at Thr57, Thr145, Ser146, and Ser130, especially catalyzed by AKT and ERK kinases, has a dual role. On one hand $p21^{Cip1/Waf1}$ is stabilized and its level in the cell is increased, but on the other hand this phosphorylation supports the relocalization of $p21^{Cip1/Waf1}$ into the cytoplasm. In the cytoplasm $p21^{Cip1/Waf1}$participates in various regulating processes; however, it undergoes degradation at the same time.

In addition to phosphorylation, another important posttranslational modification of $p21^{Cip1/Waf1}$is ubiquitylation through ubiquitine ligase such as SCF^{Skp2} (SCF = Skp1-Cullin1_F-box protein, Skp2 = S phase kinase-associated protein2), which leads to degradation of $p21^{Cip1/Waf1}$ protein in the proteasome [Bornstein et al., 2003]. The degradation of $p21^{Cip1/Waf1}$ in proteasome is caused by cooperation of many various mechanisms and they are not necessarily dependent on ubiquitylation. It was proved that the C-terminus of $p21^{Cip1/Waf1}$ protein is able to directly interact with the 20S subunit of the proteasome, bypassing the necessity of ubiquitylation. So far, the degradation of $p21^{Cip1/Waf1}$ protein was clarified in cells exposed to high UV doses. After exposure to genotoxic agent causing replication fork stalling the degradation of $p21^{Cip1/Waf1}$ is caused by ataxia telangiectasia related (ATR) activated by glycogen synthase kinase 3β (GSK-3β kinase). ATR phosphorylates $p21^{Cip1/Waf1}$ protein at ser114 [Lee et al., 2007; Soria and Gottifredi, 2010]. The degradation of $p21^{Cip1/Waf1}$ independent of ubiquitylation is probably inhibited by interaction with proliferating cell nuclear antigen (PCNA) [Cazzalini et al., 2010].

PROTEIN $p21^{Cip1/Waf1}$ IN THE NUCLEUS

In a short time after DNA damage, p53 protein is accumulated in the nucleus, whereas in non-stressed cells it mostly occurs in the cytoplasm. In the nucleus p53 works as a transcriptional factor and regulates transactivation of several proteins, including the $p21^{Cip1/Waf1}$ [Solozobova et al., 2009]. The main role of $p21^{Cip1/Waf1}$ in the nucleus is the cell cycle arrest in response to DNA damage (see Fig. 2).

Normal cells replicate their DNA once—and only once—in the S phase of the cell cycle. Further DNA replication cannot be

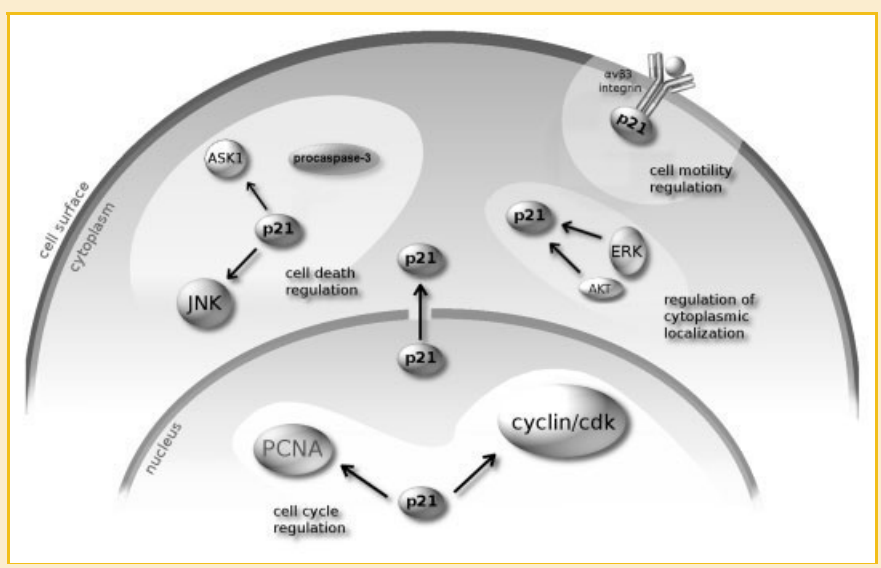

Fig. 2. Interaction of $p21^{Cip1/Waf1}$ protein depending on its subcellular localization (JNK—Jun N-terminal kinase, ASK1—apoptosis signal-regulating kinase 1, ERK—extracellular regulated MAP kinase, PCNA—proliferating cell nuclear antigen, cyklin/cdk—complex of cyclin and cyclin dependent kinase).

re-initiated before an intervening mitosis. When the DNA of a cycling cell is damaged, the cell tries to react very fast by cell cycle arrest. It enables the repair of the damaged DNA structure before the initiation of next phase. Cells lacking $p21^{Cip1/Waf1}$ or the ability to activate the expression of this protein continue with DNA synthesis and it can cause the presence of abnormal DNA [Garner and Raj, 2008]. Accumulation of $p21^{Cip1/Waf1}$ was observed in the same location as other factors of DNA reparation (Mre11, Rad50) [Cazzalini et al., 2010].

REGULATION OF G1/S CHECKPOINT BY $p21^{Cip1/Waf1}$

The G1/S checkpoint is responsible for the prevention of replication of damaged DNA. As it was mentioned earlier, $p21^{Cip1/Waf1}$ protein is able to inhibit the activity of cyclins in the G1 checkpoint and influence the transition of cells from G1 into S phase of the cell cycle. In normal conditions in early G1 phase Rb protein (retinoblastoma protein) is specificly phosphorylated by Cdk4,6/CyclinD complexes. In the end of the G1 phase the complex Cdk2/CyclinE is very important for complete phosphorylation of Rb protein, which leads the cells through the checkpoint. Fully phosphorylated Rb protein releases the E2F factor, which enables the transcription of S phase genes [Satyanarayana et al., 2008].

After DNA damage, the activity of Cdk2 is inhibited by the $p21^{Cip1/Waf1}$ protein. It leads to the accumulation of hypophosphorylated Rb protein, which can bind to the E2F factor. In this way, $p21^{Cip1/Waf1}$ protein blocks the transition between G1 and S phase and causes cell cycle arrest [Brugarolas et al., 1999; Ju et al., 2006; Cazzalini et al., 2010]. In addition, Rb protein is able to inhibit the activity of JNK kinase, which induces apoptosis after DNA damage [Garner and Raj, 2008].

Another way, in which $p21^{Cip1/Waf1}$ protein is able to influence the G1/S checkpoint is the interaction with PCNA. PCNA is a cofactor of DNA polymerases δ and ε, which are necessary for both replication and repair [Lee et al., 2009]. PCNA occurs in the cell during whole time period of cell cycle. In the end of G1 phase it is expressed in the nucleus at increased levels and in the same time relocalized from the cytoplasm into the nucleus. In addition, it serves as a marker for cells in the S phase of cell cycle. $p21^{Cip1/Waf1}$ protein reacts with the part of PCNA which binds DNA polymerases and in this way inhibits DNA replication mediated by DNA polymerase δ. The affinity of $p21^{Cip1/Waf1}$ to PCNA is much higher than the affinity of any other protein which reacts with PCNA [Li et al., 2006; Prives and Gottifredi, 2008]. In the binding of $p21^{Cip1/Waf1}$ to the PCNA protein, the balance between DNA replication, DNA repair, and cell cycle progression is regulated [Li et al., 1996].

REGULATION OF G2/M CHECKPOINT BY $p21^{Cip1/Waf1}$

Nowadays, it is well known that $p21^{Cip1/Waf1}$ protein plays an important role in the transition of cells from G2 phase of the cell cycle into mitosis. Before this transition the Cdk1/CyclinB complex is inactive, Cdk1 is phosphorylated at Tyr15 and Thr14. As the cell enters mitosis, Cdk1 is activated through the elimination of this inhibiting phosphates by Cdc24 phosphatase [Taylor and Stark, 2001]. To arrest the cell cycle in the G2/M checkpoint, Cdk1/CyclinB must occur in inactive form.

The main mechanism of cell cycle arrest in G2/M checkpoint is the regulation of Cdc25 phosphatase activity by check-point kinases 1 and 2 (Chk1, Chk2). However, it was found that the activity of the Cdk1/CyclinB complex is regulated also by interactions with $p21^{Cip1/Waf1}$ protein. The affinity of Cdk1/CyclinB complex to the $p21^{Cip1/Waf1}$ protein is low compared to other Cdk/cyclin complexes, but after the activation of G2/M point low levels of Cdk1/CyclinB complex were found binding to $p21^{Cip1/Waf1}$ protein [Nicolescu et al., 1998; Cazzalini et al., 2010].

Cdk1 can be inhibited by $p21^{Cip1/Waf1}$ protein in three ways:

(1) If $p21^{Cip1/Waf1}$ protein is in the cell in high levels, it is able to bind directly to the Cdk/cyclin complex.
(2) Cdk1 is activated by phosphorylation at Thr161 by Cdk activating kinase (CAK). CAK is formed by Cdk7, CyclinH, and Mat1 (CDK-activating kinase assembly factor). Protein $p21^{Cip1/Waf1}$ interferes with the activating phosphorylation of CAK catalyzed by Cdk1 [Taylor and Stark, 2001].
(3) The cell cycle arrest in G2 phase seem to be caused also by the interaction of $p21^{Cip1/Waf1}$ protein with PCNA protein [Cayrol et al., 1998; Taylor and Stark, 2001].

SENESCENCE

Genotoxic stress—various endogenous and exogenous agents such as ionizing radiation, UV radiation, and chemical mutagens, induce a broad spectrum of DNA damage, which leads to cell cycle arrest in the cell. This enables to gain time for damage reparation, but if the damage is too extensive to repair, the cell undergoes cell death by the process called apoptosis [Garner and Raj, 2008].

However, some cells does not undergo apoptosis after the stressors influence, but their cell cycle is permanently arrested and the cells does not proliferate. This condition is called cellular senescence. Nowadays, it is well known that replicative senescence of cells occurs after the shortening of telomere—a special nucleoprotein structure found at the ends of eucaryotic chromosomes. However, senescence can be induced prematurely after DNA damage and without relation to the telomere shortening. This condition is called stress-induced premature senescence [Vávrová and Řezáčová, 2011].

The inhibitor of the proliferation $p21^{Cip1/Waf1}$ protein has an important role in the induction of both replicative and stress-induced premature senescence. Increased expression of $p21^{Cip1/Waf1}$ protein, related to the life span of cells in tissues, was observed in corneal epithelial cells, vascular smooth muscle cells during atherosclerosis or skeletal muscles. Upregulation of this protein is also the first marker of replicative senescence induction, whereas other markers occur later, within weeks after cell cycle arrest [Ju et al., 2006]. In human fibroblasts or mesenchymal stem cells after exposure to ionizing radiation, premature senescence was induced in the cells and the induction of $p21^{Cip1/Waf1}$ protein was observed at the same time [Mirzayans et al., 2010]. However, $p21^{Cip1/Waf1}$ protein induction is connected only with the short-term cell cycle arrest and than the level is reduced to levels similar to those in non-senescent cells [Roninson, 2002; Muthna et al., 2010; Cmielova et al., 2011]. The state of permanent cell cycle arrest is maintained by another

cyclin-kinase inhibitor—$p16^{Ink4a}$ protein [Shay and Roninson, 2004].

$p21^{Cip1/Waf1}$ PROTEIN IN THE CYTOPLASM

The function of $p21^{Cip1/Waf1}$ protein in response to DNA damage probably depends on the extent of the damage. With low damage, the level of $p21^{Cip1/Waf1}$ protein increases, the protein is stabilized, induces cell cycle arrest and carries out anti-apoptotic activity, whereas after massive DNA damage levels of $p21^{Cip1/Waf1}$ protein are reduced and the cell undergoes apoptosis[3]. The presence of $p21^{Cip1/Waf1}$ protein in the nucleus is necessary for cell cycle arrest, whereas anti-apoptotic activity requires the presence of $p21^{Cip1/Waf1}$ protein in the cytoplasm (see Fig. 2).

Cytoplasmatic $p21^{Cip1/Waf1}$ protein is able to bind to the procaspase 3 (by interacting with 33 aminoacids at the N-terminus, see Fig. 1) and prevents its activation to caspase 3. This inhibits the main execution pathway of apoptosis, and the coupling of $p21^{Cip1/Waf1}$ protein to procaspase 3 blocks effectively both, receptor-mediated extrinsic pathway of apoptosis induction (membrane death receptors → initiator caspase 8 → caspase 3) and mitochondrial intrinsic pathway of apoptosis induction (p53 → proapoptotic members of bcl-2 family → apoptosom → initiator caspase 9 → caspase 3) [Coqueret, 2003; Child and Mann, 2006; Cazzalini et al., 2010].

This anti-apoptotic role is further supported by the ability of $p21^{Cip1/Waf1}$ protein to bind and inhibit pro-apoptotic kinases ASK1 (apoptosis signal-regulating kinase 1) [Cazzalini et al., 2010] and JNK [Sohn et al., 2006]. ASK1 is one of the key elements in mechanisms of stress-activated and cytokines-induced apoptosis and a member of kinase of mitogen-activated protein kinase kinase (MAPKKK). It activates two different groups MAPKK, SEK1 (MAP2K4 mitogen-activated protein kinase kinase 4, also MKK4) and MAP kinase kinase 6 (MKK6), which in turn activate stress activated protein kinases (SAPK, also known as JNK) and p38 family of MAP kinases (see Fig. 3). Increased expression of ASK1 induces apoptosis. $p21^{Cip1/Waf1}$ protein forms a complex with ASK1 and inhibits the activation of SAPK/JNK [Asada et al., 1999].

$p21^{Cip1/Waf1}$ protein and other CDK inhibitors (p27, p57) can also influence cytoskeletal factors and cell motility by inhibition of Rho cascade. Rho GTPases influence the morphology and cell motility through reorganization of actin filaments. Recently, it seems that these proteins also regulate gene expression and cell cycle process and participate also in the regulation of $p21^{Cip1/Waf1}$ protein transcription [Coleman et al., 2006]. Further, it is hypothesized that $p21^{Cip1/Waf1}$ protein is able to influence the function of integrin receptors on the cell surface and regulate cell motility [Coqueret, 2003].

CONCLUSION

$p21^{Cip1/Waf1}$ protein plays an important role in tumor initiation. On one hand it works as a tumor suppressor. It was proved, using mice models, that mice without $p21^{Cip1/Waf1}$ protein are more sensitive to tumorigenesis [Caballero et al., 2001]. The absence of $p21^{Cip1/Waf1}$ protein enables the proliferation of cells with damaged DNA and it promotes tumor progression [Gartel, 2009]. On the other hand $p21^{Cip1/Waf1}$ protein acts in an anti-apoptotic way and can behave as an oncogene. Increased expression of $p21^{Cip1/Waf1}$ protein was proved in human prostate cancer, cervical cancer, breast cancer, and in many cases upregulation of $p21^{Cip1/Waf1}$ protein correlated with the invasiveness and aggressivity of the cancer [Abbas and Dutta, 2009].

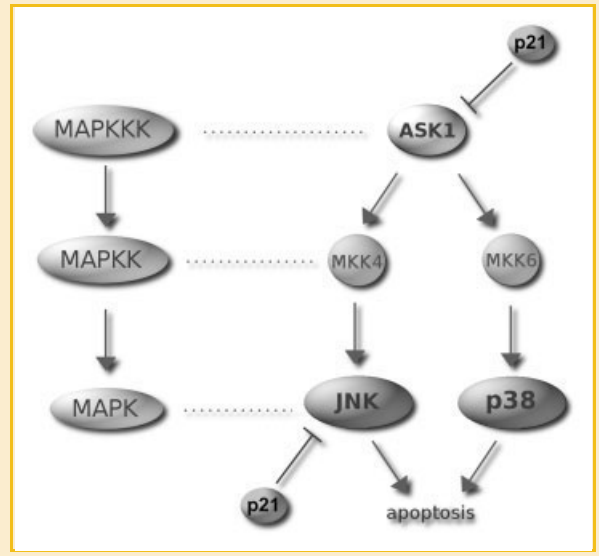

Fig. 3. Inhibition of proapototic MAPK signalization by p21 protein (MAPK—mitogen-activated protein kinase, MAPKK—kinase of mitogen-activated protein kinase, MAPKKK—kinase of mitogen-activated protein kinase kinase, ASK1—apoptosis signal-regulating kinase 1, MKK4, MKK6—MAP kinase kinase 4 and 6, JNK—Jun N-terminal kinase).

Treatment of cancer cells with different drugs often induces $p21^{Cip1/Waf1}$ and cellular senescence, which helps the cells to escape drug-induced apoptosis. It was found that in breast cancer, lung cancer or colon cancer treated by doxorubicine, the tumor cells were more susceptible to the treatment after the $p21^{Cip1/Waf1}$ protein inhibition and died [Gartel, 2009]. Better understanding of the role of $p21^{Cip1/Waf1}$ protein in cells under various conditions would help to increase efficiency of treatment of many types of cancer.

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