European Journal of Pharmacology 760 (2015) 64–71

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and cellular pharmacology

Oscillation of apoptosome formation through assembly of truncated Apaf-1

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ARTICLE INFO

Article history:
Received 25 January 2015
Received in revised form
3 April 2015
Accepted 8 April 2015
Available online 18 April 2015

Keywords:
Apaf-1
Programed cell death
Split luciferase complementary assay
Caspase activity
Apoptosis

ABSTRACT

In this study, we used spilt luciferase complementation assay strategy in order to further elucidate the main role of WD-40 repeats of Apaf-1 molecules in apoptosome formation. In the presence of ATP and cytochrome *c*, Apaf-1 monomers oligomerize and provide a platform for the activation of procaspase-9 and subsequently procaspase-3/7. For a detailed biochemical and structural investigation of Apaf-1 function and apoptosome formation, several studies have been made in recent years. However, many questions related to in vivo evaluation of this phenomenon have been persisting to answer. Some of the most important of these questions are related to WD-40 repeats at the carboxy terminus of Apaf-1 and its function in apoptosome complex formation and caspase activation. When truncated Apaf-1 molecules conjugated with luciferase fragments place in close proximity, light signal emits and real time evaluation of protein–protein interactions becomes possible. Here, we observed, for the first time, the autoassembly of truncated Apaf-1 molecules disappeared after several hours without any caspase-3/7 activation. However, we observed that, truncated Apaf-1 molecules can activate caspase-3/7 upon the induction of apoptosis via doxorubicin. Moreover, oscillation in luciferase activity upon complementation was revealed which implicates the dynamism of apoptosome formation.

1. Introduction

In humans and mice, intrinsic pathway of apoptosis is triggered by releasing of cytochrome *c*, a water soluble component of the mitochondrial electron-transport chain, into the cytosol (Green, 1998). In the intrinsic pathway, cytochrome *c* is released from mitochondria in response to stresses or lesions such as DNA damage, lack of survival signal, nutrients, or oxygen (Li et al., 1997). Cytochrome *c* and dATP-mediated oligomerization of Apaf-1 molecules form a caspase-activating complex known as apoptosome. Each Apaf-1 molecule in apoptosome complex recruits a procaspase-9 to form holoenzyme complex. Subsequently, activated caspase-9 produces caspase cascades by cleavage and activation of executioner caspase-3/7 (Zou et al., 1997). Interestingly, cytochrome *c* release, apoptosome formation, and caspase-3/7 activation are observed upon induction of both apoptosis and differentiation of mouse embryonic stem cells towards cardimyocytes (Akbari-Birgani et al., 2014).

WD repeats in Apaf-1 are involved in interaction with cytochrome *c*. Apaf-1 consists of three main functional domains: an N-terminal caspase-recruitment domain (CARD), a nucleotide-binding and oligomerization domain (NOD) and two WD40-repeats at the C-terminal of the protein (Riedl and Salvesen, 2007; Yuan et al., 2013). Caspase-recruitment domain enables Apaf-1 to be assembled with procaspase-9 and forms activated complex. WD40 repeats are responsible for the binding of cytochrome *c* (Dorstyn et al., 2002). NOD region, a dATP-binding domain, is located between CARD domain and WD-40 domains and mediates oligomerization and apoptosome formation (Genini et al., 2000).

Different Apaf-1 isoforms can be created through alternative splicing. Those isomers with seven WD-40 repeats in the first WD-40 domain can bind to cytochrome *c* and regulate the activation of procaspase-9, but those with six WD-40 domain repeats are disable (Benedict et al., 2000). Similarly, it has been shown that Apaf-1 mutants lacking the WD-40 repeats regions can be self-assembled with no activation of procaspase-3/7 (Hu et al., 1998). In order to better understand the structural dynamics and function of apoptosome complex, truncated Apaf-1 lacking the WD-40 repeats are appropriate tools.

Protein fragment complementation has emerged as a powerful tool for evaluation of protein–protein interactions (Ataei et al., 2012; Michnick et al., 2000). Firefly luciferase with high quantum yield, the

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http://dx.doi.org/10.1016/j.ejphar.2015.04.008
0014-2999/

ability of different color emissions (Hosseinkhani, 2011) and suitable structural properties is widely used for designing the biosensors and measuring noninvasive, quantitative, and real-time readout of protein–protein interactions in the cells (Nazari and Hosseinkhani, 2011). This strategy depends on division of a monomeric reporter luciferase into two separate inactive fragments whose reconstitution regenerates the enzyme activity. The interaction between proteins brings the luciferase split reporter fragments close enough to enable their reassemblage and subsequently the recovery of its native structure and activity (Binkowski et al., 2009; Paulmurugan and Gambhir, 2005). Moreover, split luciferase is an efficient tool to assay the biochemical metabolites, where a domain is inserted into an internally fragmented luciferase producing ligand binding that causes change in the photon emitted signals (Ataei et al., 2012).

We have recently presented a novel whole-cell biosensor to detect early-stages of apoptosis based on Apaf-1 oligomerization and apoptosome formation using the split luciferase strategy (Azad et al., 2014a, 2014b; Torkzadeh-Mahani et al., 2012). For this, the amino (1–416 amino acids) and carboxy fragments (395–550 amino acids) of firefly luciferase were separately fused to amino-terminal of Apaf-1. Based on this study, juxtaposition of Apaf-1 monomers and apoptosome formation was detected by emission of luminescence signal about 5 h earlier than the appearance of significant caspase-3/7 activity upon induction of apoptosis (Scheme 1). With this, we made the real time and accurate study of apoptosome formation and caspase-3/7 activation possible. Herein, for the first time, we investigated the real-time dynamism of apoptosome complex based on split luciferase strategy in doxorubicin- induced HEK293T cells. We found that the apoptosome complex composed of truncated Apaf-1 is unable to activate caspase-3/7. Surprisingly, apoptosome complex formation due to apoptosis induction is able to activate caspase-3/7 even more than control experiment.

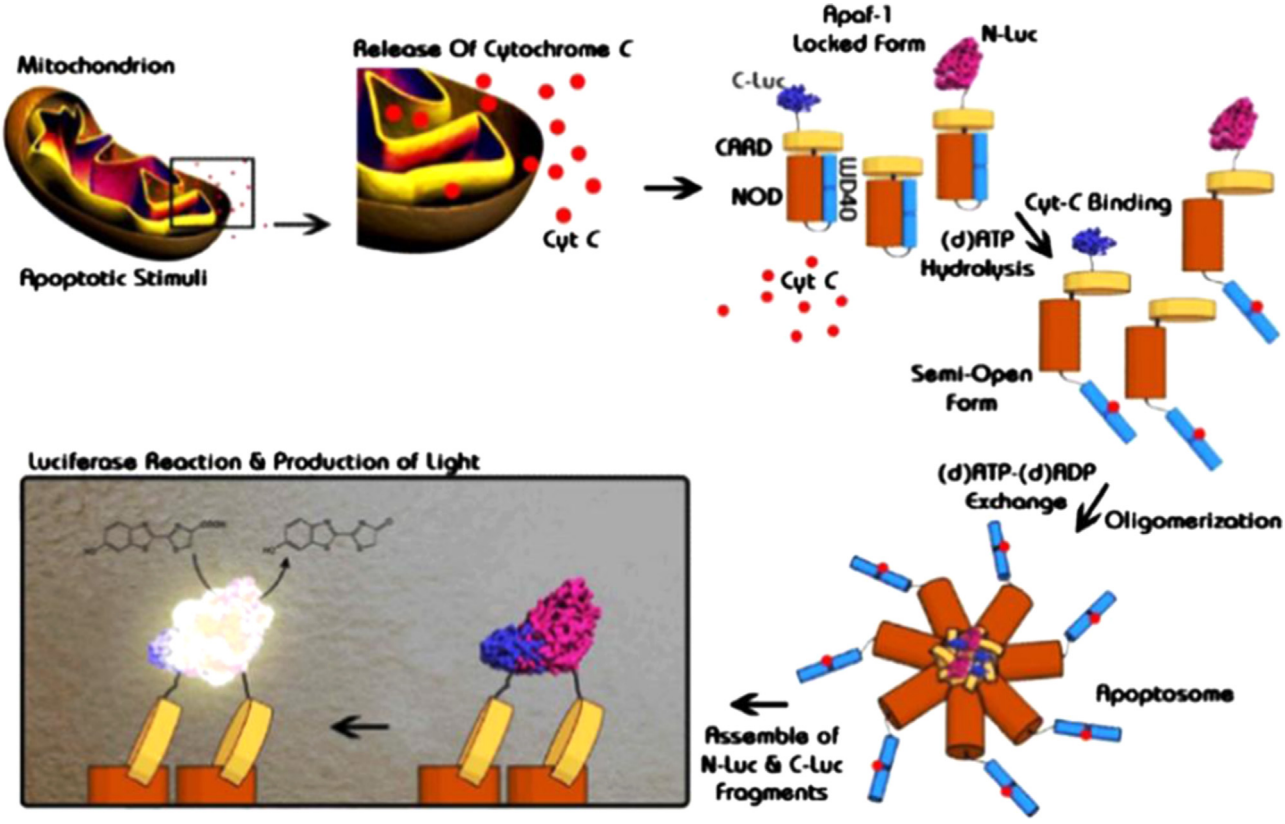
2. Materials and methods

2.1. Reagents

The following reagents were used in this study: restriction endonuclease DpnI (Fermentas), Ampicilin (Sigma), ATP (Roche), D-luciferin potassium salt (Resem BV, Netherlands), PrimeSTAR GXL DNA Polymerase (Takara Bio), a plasmid extraction kit (GeneAll Biotechnology), a gel purification kit and a polymerase chain reaction (PCR) purification kit (GeneAll Biotechnology), Dulbecco's modified eagle's medium (DMEM, high glucose; Invitrogen), fetal bovine serum (Gibco), penicillin/streptomycin solution (Invitrogen), caspase-Glo 3/7 luminescent assay (Promega)

2.2. Site-directed mutagenesis

In this study, we introduced a single point mutation in 1588 nucleotide and changed the codon of Arg to stop codon of UGA. This site of mutation is placed before WD-40 repeats and proximately at the end of nucleotide binding domain. In spite of the fact that the beginning of WD-40 repeats is not clear obviously, we decided to introduce a mutation at the end of nucleotide binding domain in order to ensure the whole of WD-40 repeats deletion. The quick change site-directed mutagenesis method (Wang and Malcolm, 1999) was used to introduce mutations. pcDNA3.1 vectors containing cDNA sequence encoding Nluc/Apaf1 and Cluc/Apaf1 were used as templates and PrimeSTAR GXL DNA Polymerase was used for amplification. Therefore, two oligonucleotide primers, each complementary to an opposite strand of pcDNA3.1 containing wild-type Apaf1 fused to luciferase fragments, used to be extended during temperature cycling by PrimeSTAR GXL DNA Polymerase and generate mutant plasmids containing staggered nicks. The quick



Scheme 1. Apoptosis biosensor mechanism of action. Upon the induction of apoptosis, cytochrome *c* is released from inter-membrane of mitochondria and causes the Apaf-1 molecule to switch from autoluck state into open form, through which apoptosome complex is formed. By formation of apoptosome, fragments of luciferase place in right position and emit luminescent signal. Reprinted from (Torkzadeh-Mahani et al., 2012) with permission from Biosensors and Bioelectronics.