Chapter 2

Enzymatic Synthesis and Purification of a Defined RIG-I Ligand

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Abstract

Receptor-based nucleic acid sensing constitutes one of the most fundamental mechanisms of our innate immune system to sense viral infection. RIG-I is a cytosolic RNA helicase that senses the presence of 5′ triphosphate RNA species, a common feature of many negative strand RNA viruses. We here describe a protocol to enzymatically synthesize and to purify a defined RIG-I ligand that can be used to study RIG-I activation in vitro and in vivo.

Key words 5′-Triphosphate RNA, RIG-ligand, Interferon induction, RNA viruses

1 Introduction

The innate immune system comprises a network of pathogen detecting receptors, which trigger intracellular defense mechanisms and the induction of innate immune responses against invading pathogens [1]. Measures against infecting viruses include inhibition of translation, RNA degradation, induction of apoptosis, and secretion of cytokines and chemokines, leading to alarming of neighboring cells and attracting immune cells [2]. In addition, the stimulation of innate immune receptors is crucial for initiation of an adaptive immune response [3]. The cytosolic immune receptor RIG-I is important for the immune response against RNA viruses [4]. It detects viruses by recognition of viral 5′ triphosphorylated RNA [5, 6]. Unlike other nucleic acid sensing innate immune receptors (e.g., TLR7, 8, or 9) RIG-I is ubiquitously expressed [7], even in transformed tumor cells [8, 9]. The fact that most of the highly pathogenic and emerging viruses are sensed by RIG-I (e.g., Influenza) highlights the relevance of this pathway. Moreover, in addition to pro-inflammatory cytokine induction, RIG-I can also trigger apoptosis [10]. By transfection of RIG-I stimulating ligands, which mimic a viral infection in the absence of
a virus, the RIG-I induced innate immune response can be exploited against tumor cells [8–11] and chronic or establishing viral infections [12–14] or for regulation of uncontrolled immune responses [15]. Moreover, employing defined RIG-I ligands can be advantageous for the analysis of pure RIG-I immune responses in absence of viral immune modulatory molecules [16].

RIG-I was found to recognize 5’ triphosphorylated RNA (pppRNA) [5, 6]. In a follow-up study, using synthetic pppRNA, we further dissected the RIG-I activating RNA ligand structure [17] (see Fig. 1): We found that RIG-I activation requires a base-paired 5’ end, which has to be part of an at least 19–20 nt base-paired RNA stretch. While blunt-ended, base-paired pppRNA (ppp-dsRNA) represents an optimal ligand for RIG-I activation, 3’ overhangs reduce and 5’ overhangs at the ppp-end abolish RIG-I activation. Indeed, crystal structure data explain the necessity of a base-paired 5’ end: The base pairing at the 5’ pp end supports a crucial stacking interaction with a phenylalanine residue in the RNA binding cleft of the C-terminal domain of RIG-I [18, 19]. In addition, in contrast to unpaired nucleotides, which allow free rotation of the following sequence, the base-paired RNA assembly stabilizes the helix in an optimal position for interaction of the adjoining phosphodiester backbone with the C-terminal domain and the helicase domain. This interaction is the prerequisite for RIG-I activation (reviewed in ref. 20). Since phage polymerase in vitro transcription (IVT) generates side products complementary to the intended transcribed sequence, any in vitro transcribed RNA (mixture), even if designed as single strand, will exhibit some RIG-I stimulating activity [17, 21]. For this reason, in previous or even in some recent studies “single stranded” triphosphorylated RNA is still considered as RIG-I ligand. However, the amount of active ligand in such in vitro transcribed “single stranded” RNA mixtures is expected to be very low and uncontrolled. To this end, we here provide a protocol to generate highly active ppp-dsRNA in a two-step protocol employing an enzymatic in vitro transcription reaction, followed by a PAGE-based purification procedure. For simplification and standardization purposes, this protocol relies on the enzymatic synthesis of a single, self-annaling hairpin RNA oligonucleotide that forms a strong intramolecular hairpin structure, thus automatically forming a dsRNA molecule (IVT4). With appropriate complexation, this molecule can be delivered into cells as a defined RIG-I stimulus for in vitro and in vivo studies [9, 11, 16, 17, 22, 23].