Identification of RIG-I CARD Interacting Cellular Proteins

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ABSTRACT

Retinoic acid inducible gene I (RIG-I) has recently been identified as a receptor capable of detecting viral RNA in the cytoplasm, subsequently interacting with VISA/MAVS/IPS-1/Cardif through its caspase activation and recruitment domain (CARD). This interaction then induces type I interferon (IFN) and cytokine expression to bring about an antiviral response. In order to elucidate novel proteins that interact with RIG-I CARD, two constructs were generated: CARD(WT)-Fc and a CARD(T55I)-Fc mutant deficient in inducing type I IFN production. Co-immunoprecipitation studies with VISA show that CARD(WT) interacts with a cleaved VISA protein. This protein was found to have a similar molecular mass as VISA 1c, which was previously found incapable of inducing type I IFN. Pull-down studies of both CARD-Fc constructs show similar protein band patterns, and the same patterns were also observed in both HuH7 and H1299 cells. A protein with apparent molecular mass of 70kDa, not detected in previous studies, was found to associate with the CARD-Fc constructs. Mass spectrometry and sequence analysis revealed it to match heat shock protein Hsp70 (accession gi|123647), suggesting a model in which Hsp70, RIG-I CARD and E3 ubiquitin ligase CHIP, might form a complex to mediate RIG-I CARD downstream signalling.

INTRODUCTION

This study sets out to identify novel endogenous proteins that interact with the CARD region of RIG-I, specifically in human cells, and postulate their role in RIG-I signalling. CARD(WT)-Fc and a CARD(T55I)-Fc mutant were generated and purified with whole cell lysates (WCL) from human cell lines H1299 (human lung cancer cell line deficient in p53) and HuH7 (human hepatoma cell line). Although there was no observable difference between the interacting proteins in both constructs, one protein bands of approximately 70kDa was present that did not interact with the Fc control. Analysis by mass spectrometry showed this protein to be Hsp70 (accession gi|123647). There were also extensive cleavage products, possibly due to cleavage of Fc domain by vaccinia virus. In addition, co-immunoprecipitation studies with VISA showed that RIG-I CARD binds to a cleaved VISA variant of apparent molecular mass 55kDa. These results help in the further characterisation of the RIG-I pathway and the roles of novel interacting partners with respect to RIG-I CARD.

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MATERIALS AND METHODS

Generation of CARD(WT)-Fc and CARD(T55I)-Fc

Figure 1. a, Structures of CARD(WT) and CARD(T55I) –Fc constructs highlighting the CARD and Fc domains. b, CARD(T55I)-Fc was constructed by a point mutation at the indicated DNA region from cytosine (C) to thymine (T), resulting in a single amino acid change from threonine (T) to isoleucine (I).

Generation of pKT- constructs

Figure 2. a, Restriction sites of BamHI, BglII and EcoRI are indicated along the CARD-Fc fusion constructs. b, Vector map of pKT0 indicating the T7 promoter site and the restriction sites. c, The ligation product of pKT0 and CARD-Fc constructs. d, The restriction sites and the complementary ends produced by each of the enzymes used.
RESULTS

Co-immunoprecipitation

Figure 2. HuH7 cells were transfected with HA-tagged VISA with Fc, CARD(WT)-Fc and CARD(T55I)-Fc. Cell lysates were subjected to immunoprecipitation with protein A-agarose. Elutes were analyzed by immunoblotting with HA and IgG antibodies. NS: non-specific band.

Silver Stain of Eluted Proteins after Pull-Down with Constructs

Figure 3. Silver stained purified Fc fusion complexes in a, H1299 b, HuH7 revealed interacting partners and multiple products of expressed constructs due to cleavage of Fc. All 10 bands (P1-P10) were analysed with mass spectrometry.

RESULTS

Significance of VISA 1c interaction

The observed interactions of CARD-Fc proteins with a protein similar to VISA 1c need to be verified by conducting co-immunoprecipitation of VISA 1c and RIG-I CARD. As previous
studies showed no interaction between RIG-I CARD and VISA 1c, it is reasoned that this interaction might be due to the presence of vaccinia virus as a possible strategy to suppress RIG-I mediated antiviral response, given that VISA 1c does not lead to IFN production due to the absence of a transmembrane domain (Lad et al., 2008).

Impact of vaccinia virus T7 expression system

The pKT – vaccinia virus T7 system was chosen initially because of its high expression level, as it is important in this study to express a high level of CARD-Fc constructs to pull-down more interacting proteins. Although vaccinia virus, a dsDNA poxvirus that replicates in the cytoplasm, has been shown to activate innate immunity through a TLR-independent pathway (Zhu, Martinez, Huang, & Yang, 2007), whether this mechanism is mediated by RIG-I has not been addressed. Also, it has been observed that infection with vaccinia virus causes a significant inhibition in host protein expression (Moss, 1968), and this might prevent the detection of endogenous interacting proteins with RIG-I CARD as the levels of expression might be too low. Thus it is impossible to make a concrete deduction from the observed interaction of Hsp70 with CARD. Also, most protein bands (P2-P10) are shown to be cleavage products of the expressed constructs, possibly the result of vaccinia virus infection, it is seen that the large scale pull-down was not efficient and failed to detect many novel interacting proteins.

Future Directions

Given the complications of the vaccinia virus expression system, future studies can be performed with another high expression vector system that does not involve vaccinia virus, and using Sendai virus (Komuro & Horvath, 2006) which has been well characterised as an efficient inducer of RIG-I dependent IFN expression to identify interacting proteins with activated RIG-I CARD. Also instead of using Fc which resulted in multiple cleavages, other tags such as Glutathione S-transferase (GST) could be used to reduce cleavage products and increase the possibility of detecting novel proteins. The observed interactions of RIG-I CARD with VISA 1c seen in figure 5c and Hsp70 in figure 6 should be validated with additional co-immunoprecipitation with these target proteins. By obtaining more information of host cell protein interactions with RIG-I CARD, we will be able to obtain a better understanding of the RIG-I pathway which is found in almost all cells in the human body, providing valuable information to efficient antiviral therapy.

REFERENCES


