The IL1 receptor accessory protein is responsible for the recruitment of the interleukin-1 receptor associated kinase to the IL1/IL1 receptor I complex

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Abstract Following interleukin-1 (IL1) stimulation, an IL1 receptor associated kinase (IRAK) is rapidly recruited to the receptor complex. However, it is not understood if IRAK is able to interact directly with the intracellular portion of the IL1-RI or if its recruitment is mediated by a different molecule. Using the yeast two-hybrid system, we have analysed possible protein-protein interactions between IRAK, IL1-RI and IL1-RAcP. We found that IRAK is able to interact with the equivalent cytoplasmic region of the IL1-RAcP but is unable to interact with the cytoplasmic region of the IL1-RI. Immunoprecipitation of the IL1-RAcP followed by Western blot analysis using anti-IRAK antibodies revealed that IRAK co-precipitated with the IL1-RAcP. We propose that, in non-stimulated cells, IRAK is bound to the IL1-RAcP and therefore, following IL1 stimulation, both molecules are recruited simultaneously to the IL1-RI complex.

Key words: Interleukin-1 receptor accessory protein; Interleukin-1 receptor associated kinase; Interleukin-1 signalling; Gene expression

1. Introduction

The interleukin-1 receptor type I (IL1-RI) belongs to a growing gene family in which members share a high degree of conservation within their intracellular portion [1]. Similarities between the cytosolic regions of *Drosophila* Toll [2,3], a member of the IL1 receptor gene family, and the mammalian IL1-RI suggested that both receptors could share a conserved signal transduction process [1,3,4]. In support of this hypothesis mutagenesis of conserved amino acids was reported to block the signal transduction cascade of both receptors [5,6]. Furthermore, in both pathways specific serine/threonine kinases, Pelle and IL1 associated kinase (IRAK), that share structural and functional similarity have also been identified [7,8]. Toll induced membrane localisation and activation of Pelle is considered to involve an adaptor-like molecule Tube [9,10]. However, since no mammalian homologue of Tube has yet been described, IL1 induced recruitment of IRAK into IL1-R complex may involve an alternative molecular mechanism.

Greenfeder et al. recently described a new IL1 receptor-like molecule, called the IL1 receptor accessory protein (IL1-RAcP), that is recruited to the type I IL1 receptor (IL1-RI) [11]. The formation of a complex including at least IL1, the IL1-RI and the IL1-RAcP appears to be essential for the initiation of IL1 signal transduction [12]. This model is supported by evidence that a cell line containing a functional IL1-RI but not expressing a full-length IL1-RAcP is unable to respond to IL1 stimulation [13,14].

The requirement of a co-receptor in the IL1 signal pathway implies that the IL1 and *Drosophila* Toll pathways could display major differences in the early stages of their signal transduction cascades. We therefore decided to study the possible protein-protein interactions among the molecules known to be present in the activated IL1-R complex.

2. Materials and methods

2.1. Constructs

Full-length IRAK and IL1-RAcP cDNAs were obtained by RT-PCR from messenger RNA isolated from HEK 293 and mouse 3T3 cell lines, respectively. The IL1-RAcP cytoplasmic region was generated by PCR from the full-length clone and spans amino acids 385–570 (IL1-RAcPct). The cytoplasmic regions of both Toll (amino acids 829–1097) and IL1-RI (amino acids 356–569) subcloned into pASCYH2 were a kind gift from Julia H.M. White (unpublished). The MyD88 full-length cDNA subcloned into the vector pGAD10 was a generous gift from Prof. J. Tschopp. Maltose binding protein (MBP) and MBP fusion proteins were generated using the vector pMAL-c2 (Biolabs) and purified by affinity chromatography on an amylose column, according to the manufacturer's instructions. The vectors pcDNA3 (Invitrogen) and pET-11a (Novagen) were utilised for IRAK expression in eukaryotic and bacterial cells, respectively. The IRAK kinase dead mutant (IRAK-kd) was generated by site-directed mutagenesis (QuikChange site-direct mutagenesis kit, Stratagene) to convert aspartic acid (D340) into asparagine. Assessment of the kinase activity of insect cell expressing IRAK-kd confirmed that the mutant IRAK was unable to auto-phosphorylate compared to insect cell extract expressing wild type IRAK (data not shown). The FLAG epitope (DYKDDDDK) was introduced into the IL1-RAcP full-length cDNA, immediately downstream of the signal peptide, Ala^{20}. The modified cDNA was subcloned into the pSG5 (Stratagene) vector, namely pSG5-F-AcP. A carboxy-terminal FLAG tagged variant of IRAK was generated by standard PCR mutagenesis.

2.2. Two-hybrid studies

The yeast Y190 (Clontech) reporter strain was utilised for the two-hybrid studies according to the supplier's instructions. Transactivation of the *his* reporter gene was assessed based on growth in presence of 25 mM 3-aminotriazole (3-AT). *LacZ* reporter gene transactivation was assayed from liquid culture using *o*-nitrophenyl-β-D-galactopyranoside as substrate.

2.3. Antibodies

Rabbit anti-human IRAK antibodies were raised against a peptide (amino acids 509–521) conjugated to keyhole limpet cyanin and peptide-specific antibodies affinity purified. Anti-MBP antibodies (Santa

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Cruz) and anti-FLAG (Kodak) were used according to the suppliers' instruction.

2.4. Stable cell line

To create a cell line stably expressing an epitope tagged version of the IL1-RAcP, HEK 293 cells were co-transfected with pSG5-AcP and pcDNA3 (Invitrogen) in 10:1 ratio, by a standard calcium-phosphate technique (Life Technology Ca-phosphate transfection system). Neomycin-resistant colonies were selected in the presence of G418 (800 μg/ml). Five independent clones were screened for FLAG tagged IL1-RAcP expression by flow cytometry (FACS). 1×10^6 cells were collected by washing twice with PBS-EDTA, 2.5% FCS, 0.1% azide (PEFA). Cells were resuspended in 100 μl of PEFA buffer and incubated in the presence of 0.5 μg of anti-FLAG antibodies for 10 min at room temperature. After three washes with PEFA buffer, cells were incubated for 5 min in the presence of anti-mouse phycoerythrin conjugated antibodies (Sigma) (1:200 dilution). Cells were washed twice in PEFA and analysed by FACS. One positive clone was identified, namely 293-F-AcP.

2.5. Immunoprecipitation

Wild type HEK 293 and 293-F-AcP cells (5×10^6) were collected by washing the cells with PBS-EDTA and collected at 1200 rpm on a bench centrifuge. Cells were resuspended in ice-cold lysis buffer [8] and sonicated for 5 s on ice. Cell debris was pelleted by centrifugation at $10000\times g$ for 15 min at 4°C. The supernatants were matched for protein content (600 μg) and 10 μg anti-FLAG antibodies coupled to agarose (Kodak) were added and incubated overnight at 4°C. In the Immunoprecipitation experiment described in Fig. 1b, 80 ng of purified MBP or MBP fusion proteins were added to a cell extract prepared from HEK 293 cells expressing a carboxy-terminal FLAG tagged version of IRAK. Immunocomplexes were collected by centrifugation and washed four times in lysis buffer. Samples were resuspended in SDS-Laemmli buffer, boiled for 4 min and subjected to SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane and specific antibody binding to the blot detected by incubation with HRP-conjugated second antibodies and ECL.

3. Results and discussion

In view of the reported homology between IRAK and Pelle we tested the capacity of IRAK to interact with Tube in yeast two-hybrid experiments [15]. However, fusions containing either the full-length (1–712) or the NH-terminal (1–208) death domain region of IRAK [16] did not interact with either full-length Tube, or a construct containing the NH-terminal, Pelle interaction region (Tube 1–257) [10] (data not shown). Since a human Tube homologue has not yet been identified the inability of IRAK to interact with *Drosophila* Tube may reflect a lack of conservation or indicate that a separate mechanism for signal transduction has evolved for the IL1-R. We therefore tested if a direct interaction between IRAK and IL1-RI or IL1-RAcP could be detected. A full-length IRAK cDNA was fused in frame to the GAL4 activation domain of pGAD-GH (pGAD-IRAK$_{712}$). The cytoplasmic regions of IL1-RI and IL1-RAcP were fused in frame to the GAL4 DNA binding domain (GAL4-BD) in pASCYH2 vector, generating pAS-IL1Rct and pAS-AcPct respectively. When co-transformed with pAS-AcPct the pGAD-IRAK$_{712}$ construct was able to transactivate both *his* and *lacZ* reporter genes but it was unable to do so if co-transformed with pAS-IL1Rct. In order to further confirm the specificity of this interaction the regions homologous to the IL1-RAcP of *Drosophila* Toll (pAS-Tollct) and of MyD88 (pAS-MyD88) were also tested. Neither construct was able to induce transactivation when co-transformed with pGAD-IRAK$_{712}$, confirming the specificity of the interaction between the IL1-RAcP cytoplasmic region and IRAK (Fig. 1a). It is meaningful to note that although IL1-RAcP, Toll, IL1-RI and MyD88 belong to the same gene family, sharing a high degree of homology through their cytoplasmic regions [17], IRAK only interacts with the IL1-RAcP.

To validate the two-hybrid interaction between, IRAK and IL1-RAcPct, a COOH-terminal FLAG tagged version of IRAK (pIRAK-FLAG) was generated and expressed in HEK 293 cells. For immunoprecipitation (IP) studies a construct encoding the IL1-RAcP cytoplasmic region (IL1-RAcPct) fused to MBP was created and the fusion protein expressed and purified from *Escherichia coli*. The MBP IL1-RAcPct fusion (MBP-AcP) or control MBP constructs were

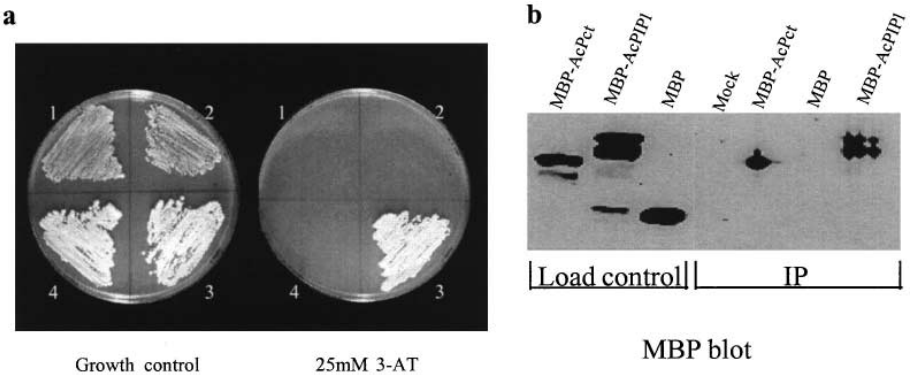

Fig. 1. IRAK interacts with the cytoplasmic region of the IL1-RAcP. a: Two-hybrid interactions. The yeast Y190 reporter strain (Clontech) was co-transformed with pGAD-IRAK$_{712}$ and IL1-RIct (1), Tollct (2), IL1-RAcPct (3) and MyD88 (4) according to the suppliers' instructions. Protein-protein interaction was detected by transactivation of *his* reporter gene in presence of 25 mM 3-AT. In the absence of 3-AT selecion double transformants are able to growth (growth control). *LacZ* reporter was also transactivated over 100-fold above control when IRAK and IL1-RAcPct were present in the same cells; determined by liquid culture β-galactosidase assay with ONPG as substrate (Clontech instruction). b: Validation of two-hybrid interaction. Anti-MBP Western blot of purified MBP fusion proteins (Load control) and proteins associated with FLAG-IRAK immunoprecipitates. Fusion proteins or MBP alone were added to the HEK extract prepared from cells expressing pIRAK-FLAG and anti-FLAG immunoprecipitates analysed by Western blotting for associated MBP proteins (IP). In addition to the cytoplasmic region of the IL1-RAcP fused to MBP (MBP-AcPct), purified MBP and MBP-AcPIP1 fusion with a novel IRAK interacting protein (manuscript in preparation) were included as negative and positive controls respectively. The IP section shows that both MBP-AcPct and MBP-AcPIP1 are immunoprecipitated from FLAG-IRAK extracts. However, anti-MBP reactive bands were not recognised in the anti-FLAG immunoprecipitates if fusion proteins were not added (Mock) or MBP alone (MBP) was added.

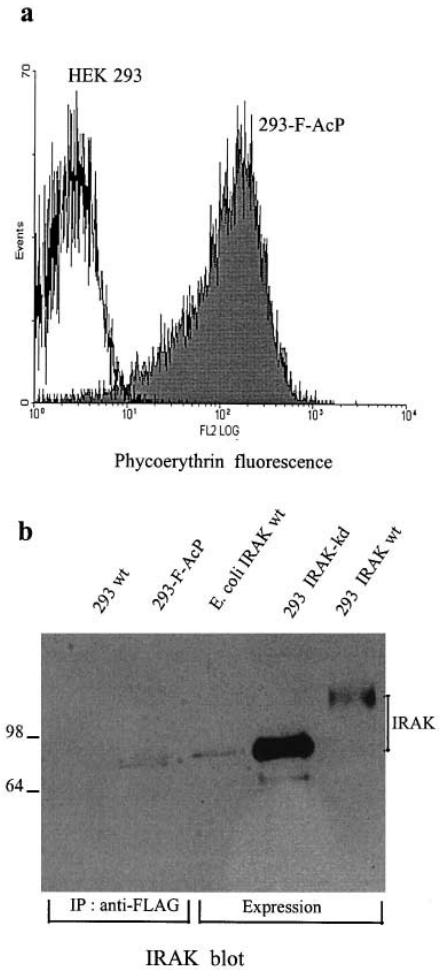

Fig. 2. IRAK is constitutively bound to the IL1-RAcP. a: 293-F-AcP cell analysis. Cell surface expression of FLAG tagged IL1-RAcP was assessed by FACS analysis using anti-FLAG antibodies on 293-F-AcP cell line and non-transfected HEK 293 cells, as described in Section 2. b: Western blot analysis with anti-IRAK antibodies. Extracts prepared from 293-F-AcP or untransfected HEK293 cells were incubated with an anti-FLAG antibody and immunoprecipitated proteins analysed by Western blotting with anti-IRAK antibodies. The IP section (lanes labelled 293wt and 293-F-AcP) shows that the anti-IRAK antibody specifically recognises a doublet present in anti-FLAG immunocomplexes prepared from 293-F-AcP, which is absent in untransfected HEK 293 cell extracts (293 wt). The molecular size of these proteins was compared with those exhibited by wild type IRAK expressed in *E. coli* and wild type IRAK (IRAK-wt) or an IRAK kinase domain mutant (IRAK-kd), expressed in 293 cells.

incubated with the HEK 293 cell extract and IRAK-FLAG immunoprecipitated using anti-FLAG antibodies (Kodak). Immunocomplexes were analysed by western blotting using anti-MBP antibodies (Santa Cruz). This experiment confirmed that IRAK can associate with the IL1-RAcP (Fig. 1b, IP section).

To establish whether IRAK recruitment follows formation of the IL1/IL1-RI/IL1-RAcP complex or if IRAK is bound constitutively to the IL1-RAcP the existence of IL1-RAcP-IRAK complex was investigated. For these studies an HEK 293 cell line, stably expressing a full-length amino-terminally FLAG tagged IL1-RAcP (named 293-F-AcP), was generated. FACS analysis using an anti-FLAG antibody demonstrated cell surface localisation of the expressed epitope tagged receptor (Fig. 2a). Anti-FLAG antibodies were used to immunoprecipitate the epitope tagged IL1-RAcP from 293-F-AcP cell extracts and an equivalent extract prepared from untransfected HEK 293 cells was used as a control. To detect the presence of IRAK, immunocomplexes were analysed by Western blotting using an anti-IRAK peptide specific antibody. Two proteins of about 76 kDa were immunoprecipitated with anti-FLAG antibody from the 293-F-AcP cell extract were not detected when extracts from untransfected cells were used (Fig. 2b, lanes 293-F-AcP and 293-wt). We compared the mobility of these proteins to those exhibited by IRAK which had been expressed in *E. coli* and HEK 293 cells (Fig. 2b, lanes E. coli IRAK wt and 293 IRAK wt). An extract prepared from HEK 293 cells expressing a IRAK-kd mutant was also included in the comparison (lane 293 IRAK-kd). As shown in Fig. 2b, the upper band present in the immunocomplex derived from the 293-F-AcP cell line comigrates with both IRAK-wt expressed in *E. coli* and IRAK-kd expressed in eukaryotic cells. IRAK-wt expressed in HEK-293 cells migrates with reduced mobility suggesting that this protein undergoes post-translational modification; the presence of a smaller immunoreactive band could be due to post translational modifications. Evidence that the kinase domain mutant of IRAK does not exhibit this mobility shift suggests that the modification may involve IRAK-dependent phosphorylation. We therefore propose that in the absence of IL1 stimulation, IRAK is associated with the cytoplasmic region of the IL1-RAcP.

Our finding that IRAK is constitutively associated with the IL1-RAcP could suggest a model for signal transduction common to other cytokine receptors, in which one chain is responsible for the ligand binding and another is required to activate downstream cytoplasmic signalling events. However, since the IL1-RI cytoplasmic region is essential for the activation of IL1 signal transduction, signalling molecules associated with this chain may also be important [5]. In fact, molecules with signal transducing features [18] and molecules with kinase activity [19] have been described to associate with the IL1-RI. These findings support the idea that the cytoplasmic regions of both IL1-RI and IL1-RAcP play major roles in inducing the activation and regulation of IL1 signal transduction.

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