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Cellular trafficking of the IL-1RI-associated kinase-1 requires intact kinase activity ☆

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Abstract

Upon stimulation of cells with interleukin-1 (IL-1) the IL-1 receptor type I (IL-1RI) associated kinase-1 (IRAK-1) transiently associates to and dissociates from the IL-1RI and thereafter translocates into the nucleus. Here we show that nuclear translocation of IRAK-1 depends on its kinase activity since translocation was not observed in EL-4 cells overexpressing a kinase negative IRAK-1 mutant (EL-4$^{IRAK\text{-}1\text{-}K239S}$). IRAK-1 itself, an endogenous substrate with an apparent molecular weight of 24 kDa (p24), and exogenous substrates like histone and myelin basic protein are phosphorylated by nuclear located IRAK-1. Phosphorylation of p24 cannot be detected in EL-4$^{IRAK\text{-}1\text{-}K239S}$ cells. IL-1-dependent recruitment of IRAK-1 to the IL-1RI and subsequent phosphorylation of IRAK-1 is a prerequisite for nuclear translocation of IRAK-1. It is therefore concluded that intracellular localization of IRAK-1 depends on its kinase activity and that IRAK-1 may also function as a kinase in the nucleus as shown by a new putative endogenous substrate.

Keywords: IL-1 signaling; IRAK-1 recruitment; Nuclear localization; Cellular trafficking; Redox regulation; Menadione

The function of the serine/threonine kinase IRAK-1 in the IL-1- and the Toll-like-receptor (TLR)-signaling pathway is still under debate. Two recent publications have shown that IRAK-1 translocates into the nucleus [1] and possesses gene regulatory function by interaction with Stat3 and the IL-10-promoter [2].

The signaling complex formed upon binding of IL-1 to the IL-1RI consists of different adapter proteins and kinases. By now, the following key events contribute to the formation of the IL-1RI signaling complex (reviewed in [3]): the initial signal transduction depends on the presence of the IL-1R-accessory protein (IL-1R-AcP) which heterodimerizes with the IL-1RI and interacts with the IL-1RI associated serine/threonine kinase IRAK-1; the binding of IRAK-1 to the IL-1RI and IL-1R-AcP is facilitated by the adapter protein MyD88; IRAK-1 moves to the receptor in a complex with tollip which binds to the AcP and allows IRAK-1 to interact with MyD88; IRAK-1 becomes phosphorylated within the receptor complex and regulates its own availability as an adapter molecule by sequential (auto-)phosphorylation [4]. At the IL-1RI, IRAK-1 becomes hyperphosphorylated first on Thr-209 and Thr-387, presumably by IRAK-4, and then autonomously. Upon phosphorylation, IRAK-1 is released from the IL-1RI complex and can either be ubiquitinated and degraded by the proteasome or translocates into the nucleus. Either IRAK-1 itself or other associated kinase(s) phosphorylate a yet unidentified endogenous target with an apparent molecular weight of 60 kDa (p60). Also phosphatidylinositol (PI) 3-kinase is recruited to a specific site in the activated IL-1RI. Downstream signaling by the activated IL-1RI complex is

☆ *Abbreviations:* IL-1, interleukin-1; IL-1RI, IL-1 receptor type I; IRAK-1, IL-1RI associated kinase type 1.

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mediated by a member of the TNF-associated factor family, TRAF6, and involves the activation of the TGF-β-activated kinase (TAK), and I-κB kinase (IKK). The latter enables activation of the transcription factor NF-κB and its translocation into the nucleus by phosphorylation of the inhibitor protein I-κB. Activation of various genes by NF-κB is the consequence of cellular activation by IL-1.

IRAK-1 has been the first kinase to be identified in the IL-1 pathway [5,6], it, however, remains uncertain whether IRAK-1 acts as a kinase or an adapter protein. IRAK-M from monomyeloic cells [7] and IRAK-2 [8] do not exhibit any kinase activity. The kinase activity of IRAK-1 has been shown to be dispensable for the IL-1-mediated activation of NF-κB and production of IL-2 in T cells [9–11]. Even complete deletion of the kinase domain did not inhibit the IL-1-mediated activation of JNK or NF-κB [12]. The kinase activity, however, was reported to be necessary to maintain the ability of IRAK-1 to assemble into high molecular weight particles for which the conserved Thr-66 seemed to be required [13]. Complexes of endogenous IRAK-1, which are also present in unstimulated cells, were discussed as a form of storage of the inactive protein [13]. IRAK-1 was also detected within high molecular mass complexes along with the NF-κB essential modulator (NEMO) and I-κB kinase β [14].

Several authors have shown a receptor-mediated trafficking of IL-1 into the nucleus [15–18]. It is not clear whether nuclear localization of IL-1RI and IL-1β is essential for IL-1 signaling in general [15,19]. Transport of IL-1 into the nucleus correlates with impaired endothelial cell growth and expression of some IL-1α-inducible genes [20] and regulates the migratory capacity of human endothelial cells [21]. The ligand IL-1 [15–18,22], the IL-1RI [15,23], and the receptor-associated PI3-kinase [24] were detected in the nucleus upon IL-1 stimulation. The translocation of receptor-bound IL-1 was claimed to contribute to or to be essential for gene transactivation [15,18]. The potential relevance of trafficking of IL-1RI complex signaling components is further supported by the detection of two proteins with nucleic acid binding sequence characteristics (p38, p67) in the immuno-precipitated IL-1RI complex [25] and putative nuclear localization sequences (NLS) in the cytoplasmic domain of the IL-1RI [15,26], in the PI3-kinase [24], in mature IL-1β [17], and in the precursor but not the mature form of IL-1α [20,27].

Formation of the IL-1RI complex requires a regulated sequence of protein recruitment. One possibility to regulate activity and interaction of the proteins involved is modification of protein thiols, either by direct interaction with reactive compounds or by changes in the cellular redox state (reviewed in [28]). IL-1 signaling involves the production of reactive oxygen species [29–31]. Modification of protein thiols was shown to inhibit responses to IL-1 such as phosphorylation of substrates within the IL-1RI complex [32,33], activation of the transcription factor NF-κB [32], and the expression of adhesion molecules [34]. Already the recruitment of IRAK-1 to the receptor is a redox-sensitive step [35]. We, therefore, made use of thiol modifying agents to inhibit the recruitment of IRAK-1 to the IL-1RI and demonstrate that this transient association of IRAK-1 to the receptor is necessary for its subsequent transport into the nucleus. We here provide data that the kinase activity is indispensable for nuclear translocation of IRAK-1 and that there are endogenous substrates for IRAK-1 in the nucleus.

Materials and methods

Cell culture and treatment. The murine thymoma cell line EL-4 6.1 stably overexpressing IRAK-1 (EL-4^{IRAK-1}) and a kinase negative mutant of IRAK-1 (EL-4$^{IRAK-1-K239S}$) were kind gifts of J. Knop and M.U. Martin, Medical School Hannover, Germany. Overexpressed IRAK-1 has been FLAG-tagged in these cells. Cells were cultured in RPMI 1640 containing 5% FCS (Biochrom), 2 mM L-glutamine, 2 mM penicillin/streptomycin (Invitrogen), 50 μM β-mercaptoethanol, and 0.4 mg/ml geneticin (Calbiochem). Cells were washed two times with medium lacking FCS and then left untreated or stimulated with 5 ng/ml recombinant human (rh)IL-1β (GBF, Braunschweig, Germany) at 37 °C for up to 2 h at a cell density of 5×10^6 cells/ml.

Cell lysis and preparation of nuclear extracts. After stimulation with IL-1, 2×10^7 cells were washed two times with PBS and lysed in 1 ml lysis buffer containing 1% Brij97 (Sigma), 50 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, and the protease inhibitors pepstatin (1 μg/ml) and pefabloc (1 mM, Roth) at 4 °C for 30 min. Lysates were centrifuged for 10 min at 10,000*g* and postnuclear supernatants were used for immunoprecipitation experiments. For preparation of nuclear extracts cells were washed in PBS and resuspended in 0.1% Brij97, 50 mM NaCl, 50 mM Tris, pH 7.4, at 4 °C for 20 min. After centrifugation, pellets were washed twice and nuclear protein was extracted with high salt buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). Nuclear extracts were also prepared by pottering of the cells and ultracentrifugation of proteins through 30% sucrose and the purity was controlled by measurement of marker enzymes as described previously [1]. Since the results shown here were identical for both methods, the shorter preparation protocol was used.

Immunoprecipitation and in vitro kinase assay. For immunoprecipitation postnuclear supernatants of Brij97 lysates or 150 μg nuclear protein was incubated with 0.35 μg anti-IRAK-1-antibody (UBI, Lake Placid, USA) or 1 μg anti-IL-1RI-antibody (Pharmingen, Heidelberg, Germany) and 30 μl of a protein G–Sepharose slurry at 4 °C overnight. Immunoprecipitates were washed and centrifuged three times in lysis buffer (see above) and three times in kinase buffer consisting of 20 mM Hepes, pH 6.5, 100 mM NaCl, 5 mM $MnCl_2$, and 5 mM $MgCl_2$. Pellets were resuspended in 45 μl kinase buffer and in vitro kinase assay was performed by adding 1 μCi [^{32}P-γ]ATP for 10 min at room temperature. The kinase reaction was stopped by addition of kinase buffer containing 40 mM EDTA. Subsequently, immunoprecipitates were washed three times with the same buffer and boiled in Laemmli buffer for 5 min. Proteins were separated on 7.5% or 12.5% SDS–polyacrylamide gels as indicated in the figures. Gels were dried and phosphorylated proteins were made visible by autoradiography using Kodak X-omat films. Alternatively, gels were subjected to Western blot detection.

Western blot analysis. Washed immunoprecipitates were separated on SDS–polyacrylamide gels and blotted to nitrocellulose (2 h,

1.2 mA/cm^2, 4 °C). The blots were blocked in Tris-buffered saline containing 0.1% Tween 20 (TTBS) with 5% non-fat dried milk at room temperature for 1 h and subsequently incubated with an anti-IRAK-1 antibody in TTBS (1 μg/ml, Santa Cruz Biotechnology). Western blot analysis was performed with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:50,000, Roche). Proteins were visualized by chemiluminescence imaging (using Supersignal West Dura, Perbio) with the Fuji LAS1000-CCD-camera system.

Results

IRAK-1 is translocated into the nucleus and phosphorylates exogenous substrates

Nuclear translocation of IRAK-1 was investigated in EL-4 6.1 cells stably overexpressing FLAG-tagged IRAK-1 (EL-4^{IRAK-1}) in which the amount of IRAK-1 is highly increased in comparison to wild type EL-4 cells (Fig. 1A) [36]. The unphosphorylated form of IRAK-1 is detectable in a Western blot as a band at 80 kDa (Fig. 1A, lanes 1 and 3), whereas phosphorylated IRAK-1 is visible as a broad band ranging from 80 to 100 kDa which results from multiple phosphorylation events indicating that overexpression of IRAK-1 is preferentially apparent in stimulated cells (Fig. 1A, lane 4).

Nuclear IRAK-1 can be phosphorylated in an in vitro kinase assay as shown in IRAK-1-immunoprecipitates of nuclear extracts from cells after 90–120 min of IL-1 stimulation (Fig. 1B, lanes 3, 4, 7, and 8). Phosphorylated IRAK-1 is visible at a molecular weight of 80–100 kDa indicating multiple phosphate residues. The degree of IRAK-1-phosphorylation visible as shift in molecular weight is higher in the cytoplasm/membrane fraction (Fig. 1A) than in the nuclear fraction (Fig. 1B). IRAK-1 becomes (auto-)phosphorylated in the in vitro kinase assay either: (i) by itself or (ii) by a kinase co-precipitating and co-migrating into the nucleus with IRAK-1 upon IL-1-stimulation (iii) or by a kinase which is already located in the nucleus.

Five minutes of IL-1-stimulation was not sufficient for IRAK-1 to translocate and to detect a kinase activity in the nucleus (Fig. 1B, lanes 2 and 6) which is also not visible in unstimulated cells (Fig. 1B, lanes 1 and 5). In contrast, IRAK-1 is highly phosphorylated in the cytoplasm after short IL-1-stimulation times (Fig. 1A). Phosphorylation of IRAK-1 in nuclear extracts was more pronounced in anti-IRAK-1- (Fig. 1B, lanes 3 and 4) than in anti-FLAG-immunoprecipitates of EL-4^{IRAK-1} cells (Fig. 1B, lanes 7 and 8), which have been prepared as control to visualize only overexpressed, but not endogenous IRAK-1 in these cells. This might result from a distinct level of endogenous IRAK-1 in these cells and/or from different affinities of the antibodies used for immunoprecipitation of IRAK-1.

To confirm that IRAK-1 possesses kinase activity in the nucleus, histone H1, and myelin basic protein

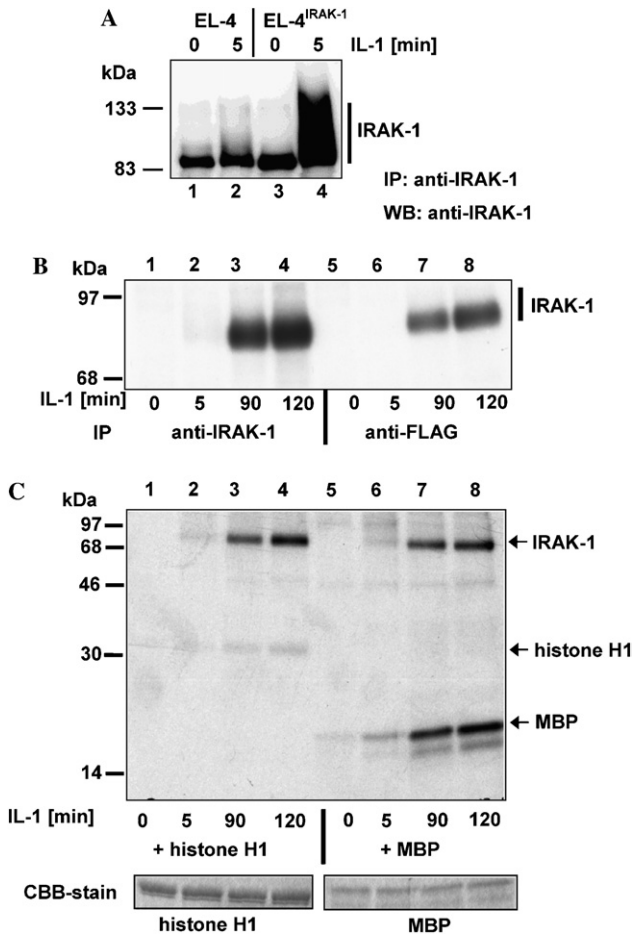

Fig. 1. Appearance of IRAK-1 in the nucleus upon IL-1 stimulation and phosphorylation of exogenous substrates. (A) 2×10^7 EL-4 resp. EL-4^{IRAK-1} cells were stimulated with IL-1β (5 ng/ml) for the indicated times and IRAK-1 was immunoprecipitated (IP) from postnuclear supernatants of Brij97 lysates with an anti-IRAK-1-antibody (ab). Precipitates were separated on a 7.5% SDS gel, immunoblotted, and developed with an anti-IRAK-1-ab (WB, Western blot). IRAK-1 is marked and molecular weight standards are indicated in kDa on the left. (B) 2×10^7 EL-4^{IRAK-1} cells were stimulated with IL-1β for the indicated times and proteins were immunoprecipitated (IP) from 150 μg nuclear extracts with an anti-IRAK-1-ab or an anti-FLAG-ab. Precipitates were subjected to an in vitro kinase assay using [^{32}P-γ]ATP. Thereafter, proteins were separated on 7.5% SDS–gels and IRAK-1 was made visible by its (auto-)phosphorylation. IRAK-1 is marked and molecular masses are indicated in kDa on the left. (C) Alternatively, the in vitro kinase assay was performed with anti-IRAK-1-immunoprecipitates of nuclear extracts using histone H1 (2.5 μg) and myelin basic protein (MBP, 5 μg) as exogenous substrates. Shown is an autoradiograph of a 12.5% SDS–gel. Phosphorylated IRAK-1 (80–100 kDa), histone H1 (33 kDa), and MBP (21 kDa) are marked with arrowheads. The Coomassie brilliant blue (CBB) staining of the exogenous substrates is shown as control for equal amounts of protein. Molecular masses are indicated in kDa.

(MBP) were added to the IRAK-1-immunoprecipitates from nuclear extracts and used as exogenous substrates in the in vitro kinase assay. As shown in Fig. 1C histone H1 and MBP were only phosphorylated by IRAK-1-immunoprecipitates from nuclear extracts of long-term

stimulated EL-4^{IRAK-1} cells (Fig. 1C, lanes 3 and 4 (histone H1), resp. 7 and 8 (MBP)), but not in unstimulated (Fig. 1C, lanes 1 and 5) or short-term (5 min) stimulated cells (Fig. 1C, lanes 2 and 6) where nuclear translocation of IRAK-1 has not taken place yet. The phosphorylation of exogenous substrates correlated with the phosphorylation of IRAK-1 detectable at 80–100 kDa. For visualization of histone H1 and MBP a 12.5% SDS–PAGE had been used which prevented the band broadening of IRAK-1 due to phosphorylation as visible for nuclear extracts in a 7.5% gel (Fig. 1B). As loading control for equal amounts of substrates, gels were stained with Coomassie brilliant blue, histone H1 and MBP were excised and depicted below (Fig. 1C, CBB-stain). These results indicate that IRAK-1 translocated into the nucleus indeed possesses kinase activity.

Nuclear localization of IRAK-1 depends on its kinase activity

Mutation of lysine 239 into serine in the ATP-binding pocket destroys the kinase activity of IRAK-1. To investigate whether the kinase activity is necessary for nuclear localization of IRAK-1, we compared the amounts of nuclear located IRAK-1 after prolonged IL-1 stimulation times (90 min resp. 120 min) in EL-4^{IRAK-1} and EL-4$^{IRAK-1-K239S}$ cells in an anti-IRAK-1-Western blot. Without kinase activity, IRAK-1 protein was not detectable in nuclear extracts in contrast to those cells overexpressing kinase-active IRAK-1 (Fig. 2A, compare the right part (EL-4$^{IRAK-1-K239S}$) with the left part (EL-4^{IRAK-1}) of the figure).

Additionally, IRAK-1 was precipitated from the cytoplasm/membrane fraction using an anti-IRAK-1-antibody. In vitro kinase assays of the precipitates did not detect any IRAK-1 kinase activity in unstimulated EL-4^{IRAK-1} or EL-4$^{IRAK-1-K239S}$ cells (Fig. 2B, lane 1, EL-4^{IRAK-1} and EL-4$^{IRAK-1-K239S}$ cells). Upon stimulation with IL-1 IRAK-1 becomes phosphorylated (Fig. 2B, lanes 2–4, EL-4^{IRAK-1}, and EL-4$^{IRAK-1-K239S}$ cells). The degree of phosphorylation was much higher in kinase-active EL-4^{IRAK-1} cells than in the mutant cells. The faint phosphorylation of overexpressed IRAK-1 in EL-4$^{IRAK-1-K239S}$ cells might have been derived from the activity of precipitated endogenous IRAK-1 or coprecipitated IRAK-4 in the cytosol.

Mutant IRAK-1 was not present in nuclear extracts (Fig. 2A) and, therefore, did not exert any kinase activ-

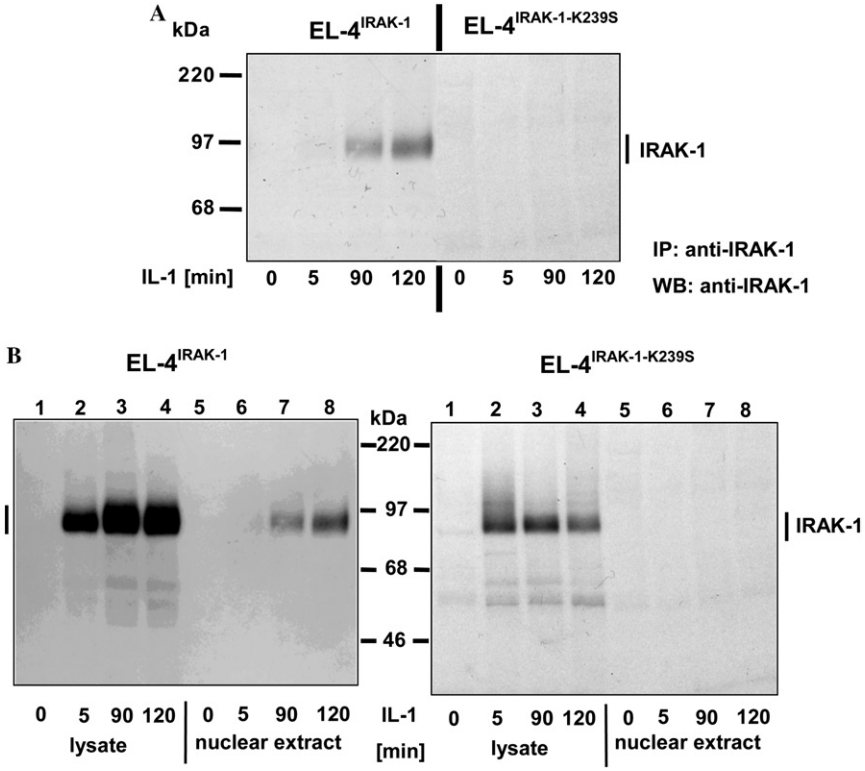

Fig. 2. Nuclear localization of IRAK-1 depends on its kinase activity. (A) EL-4^{IRAK-1} and EL-4$^{IRAK-1K239S}$ cells were stimulated with IL-1β for the indicated times. IRAK-1 was immunoprecipitated from nuclear extracts using an anti-IRAK-1-ab. Precipitates were separated on a 7.5% SDS–gel, immunoblotted, and developed with an anti-IRAK-1-antibody. IRAK-1 is marked and molecular weight standards are indicated in kDa on the left. (B) After IL-1 stimulation for the indicated times, EL-4^{IRAK-1} and EL-4$^{IRAK-1-K239S}$ cells were either lysed in Brij97-buffer and postnuclear supernatants (lysates) or, alternatively, nuclear extracts were prepared. Both types of samples were used for immunoprecipitation of IRAK-1 and applied to an in vitro kinase assay. IRAK-1 was made visible by its (auto-)phosphorylation on 7.5% SDS–gels. Phosphorylated IRAK-1 and molecular masses are indicated.

ity as expected (Fig. 2B, right figure, lanes 5–8). Also the amount of endogenous, kinase-active IRAK-1 in EL-4$^{IRAK-1-K239S}$ cells was not sufficient to detect either phosphorylated kinase-inactive IRAK-1 or phosphorylated endogenous IRAK-1 in the nucleus. In contrast, phosphorylation of IRAK-1 in nuclear extracts of kinase-active EL-4^{IRAK-1} cells was detectable here as already shown in Fig. 1 (Fig. 2B, left figure, lanes 7 and 8).

The degree of IRAK-1 phosphorylation was higher in the cytosolic/membranous fraction in comparison to the nuclear fraction (compare Fig. 2B, left part, lanes 3 and 4 with lanes 7 and 8). This might indicate a different total amount and phosphorylation status of IRAK-1 in these cell compartments. Hence, nuclear translocation of IRAK-1 appears to depend on its kinase activity.

Recruitment of IRAK-1 to the IL-1RI is necessary for its nuclear localization

To elucidate whether recruitment of IRAK-1 is necessary for its subsequent translocation into the nucleus, we first compared the amount of IRAK-1 which is recruited to the IL-1RI upon ligand binding in EL-4^{IRAK-1} (Fig. 3A, lane 2) and EL-4$^{IRAK-1-K239S}$ cells (Fig. 3A, lane 4) in a coprecipitation experiment. In unstimulated cells, IRAK-1 is not associated to the IL-1RI (Fig. 3A, lanes 1 and 3). The quantity of IRAK-1 protein associated to the IL-1RI upon ligand binding was higher in kinase-active EL-4^{IRAK-1} cells than in the mutant cells. Since IRAK-1 was only detectable as a shifted band caused by multiple phosphorylation events, the weak amount of shifted IRAK-1 in EL-4$^{IRAK-1-K239S}$ cells might have been derived from the kinase activity of coprecipitated endogenous IRAK-1 or IRAK-4 in the cytosol which is not altered in the kinase-negative cell line. Hence, in both cell lines IRAK-1 is recruited to the IL-1RI upon ligand binding, but phosphorylated and shifted in molecular weight to a different extent.

To further corroborate the necessity of IRAK-1 recruitment to the IL-1RI for its subsequent nuclear translocation, we additionally made use of the inhibition of IRAK-1 recruitment by the thiol modifying agent menadione [32]. Treatment of EL-4^{IRAK-1} cells with menadione completely abolished the recruitment of IRAK-1 to the receptor (Fig. 3B) which is caused by the blockade of distinct cysteine-residues in the IRAK-1 protein and was shown to be reversible in a glutathione-dependent manner [32]. IRAK-1 was not present in nuclear extracts of unstimulated EL-4^{IRAK-1} cells or cells stimulated with IL-1 for 5 min only and was, therefore, not detectable by its kinase activity (Fig. 3C, lanes 1, 2, 5, and 6). Phosphorylated IRAK-1 was only detectable after long IL-1-stimulation times without menadione pretreatment of the cells indicating that this thiol modifying agent inhibited nuclear translocation of IRAK-1 (Fig. 3C, lanes 7 and 8 versus lanes 3 and 4).

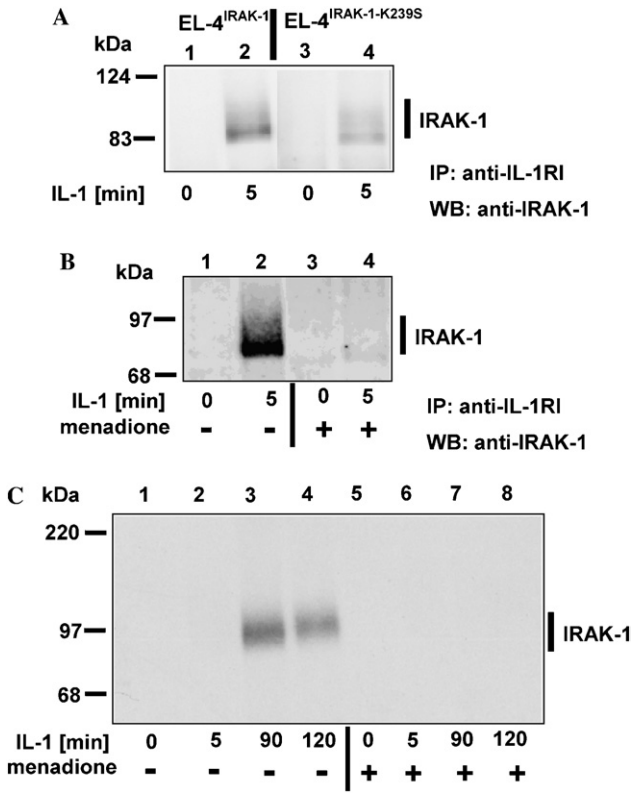

Fig. 3. IRAK-1 has to be recruited to the IL-1RI prior to nuclear localization. (A) EL-4^{IRAK-1} and EL-4$^{IRAK-1K239S}$ cells were left untreated or stimulated with IL-1β for 5 min. Proteins were immunoprecipitated from postnuclear supernatants of Brij97 lysates with an anti-IL-1RI-ab. IRAK-1 was detected by Western blotting of a 7.5% SDS gel using an anti-IRAK-1-antibody. IRAK-1 is marked and molecular weight standards are indicated in kDa on the left. (B) EL-4^{IRAK-1} cells were preincubated with or without menadione (50 μM) for 15 min at 37 °C as indicated. Cells were stimulated with IL-1β for the times indicated and IRAK-1-protein was co-precipitated with the IL-1RI and visualized as described in (A). (C) EL-4^{IRAK-1} cells were treated as described in (B), IRAK-1 protein immunoprecipitated from nuclear extracts with an anti-IRAK-1-antibody and applied to an in vitro kinase assay. Phosphorylated IRAK-1 was visualized as described in Fig. 2B and is marked. Molecular masses are indicated in kDa on the left.

Hence, reduced thiols in the IRAK-1 protein are a prerequisite for its recruitment to the IL-1RI [35] which itself is necessary for its subsequent translocation into the nuclear compartment.

IRAK-1 phosphorylates an endogenous substrate in the nucleus

We next tested whether IRAK-1 phosphorylates endogenous substrates in nuclear extracts. IRAK-1 and a 24 kDa protein (indicated as p24) were phosphorylated in anti-IRAK-1-immunoprecipitates of nuclear extracts prepared from EL-4^{IRAK-1} cells stimulated with IL-1 for 120 min (Fig. 4, lane 3). Phosphorylated p24 is not visible in kinase assays of IRAK-1-immunoprecipitates from nuclear extracts of unstimulated cells or those

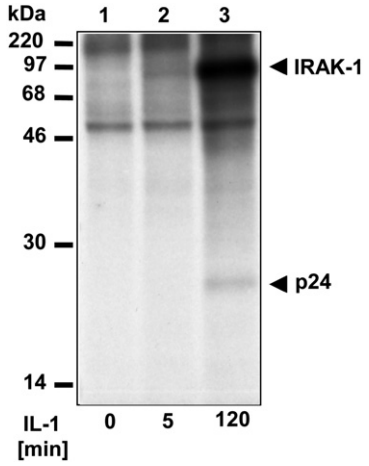

Fig. 4. Phosphorylation of an endogenous nuclear substrate by IRAK-1. EL-4^{IRAK-1} cells were stimulated with IL-1β for the indicated times. IRAK-1 was immunoprecipitated from 150 μg nuclear extract, applied to an in vitro kinase assay, and made visible by its (auto-) phosphorylation on a 12.5% SDS–gel. Phosphorylated IRAK-1 and the endogenous substrate p24 are marked with arrowheads. Molecular masses are indicated in kDa on the left.

treated only 5 min with IL-1 (Fig. 4, lanes 1 and 2). p24 must, therefore, have been phosphorylated by IRAK-1 in the nucleus and/or translocated with IRAK-1 from the cytosol into the nucleus.

Phosphorylation of p24 depends on kinase-active IRAK-1

To ensure that p24 is a specific target of nuclear IRAK-1, we compared p24 phosphorylation in EL-4^{IRAK-1} and EL-4$^{IRAK-1-K239S}$ cells. IRAK-1 immunoprecipitated from nuclear extracts phosphorylated p24 only in EL-4^{IRAK-1}, but not in EL-4$^{IRAK-1-K239S}$ cells (Fig. 5). Obviously, the kinase activity of IRAK-1

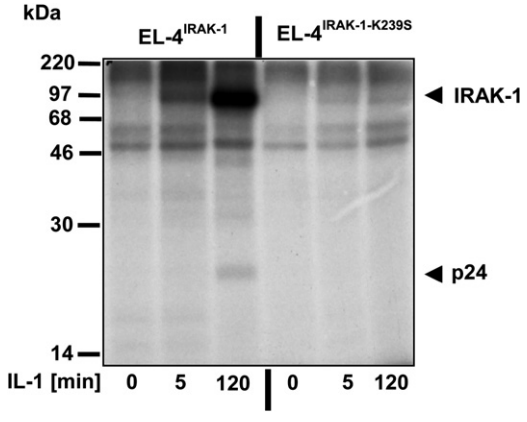

Fig. 5. Phosphorylation of p24 depends on the kinase activity of IRAK-1. EL-4^{IRAK-1}, and EL-4$^{IRAK-1-K239S}$ cells were stimulated with IL-1β for the indicated times. IRAK-1 was immunoprecipitated from 150 μg nuclear extract and visualized as described in Fig. 4. Phosphorylated IRAK-1 and the endogenous substrate p24 are marked with arrowheads. Molecular masses are indicated in kDa on the left.

and not that of any other nuclear located kinase is responsible for the phosphorylation of the putative endogenous target p24.

p24 is associated to IRAK-1

To test whether p24 translocates into the nucleus together with IRAK-1 or if it is already present in the nuclear compartment, in vitro kinase assays of IRAK-1-immunoprecipitates of postnuclear lysates were compared with those of nuclear extracts (Fig. 6, anti-IRAK-1-IP). Additionally, the same experiment was performed in IL-1RI-immunoprecipitates of both cell compartments (Fig. 6, anti-IL-1RI-IP). The endogenous substrate p24 was not phosphorylated in IL-1RI-immunoprecipitates of nuclear extracts or postnuclear lysates of EL-4^{IRAK-1} cells, whereas phosphorylated IRAK-1 was associated to the receptor and to the endogenous 60 kDa substrate (Fig. 6, left part).

Phosphorylated p24 was visible in IRAK-1-immunoprecipitates of nuclear extracts after prolonged IL-1-stimulation and also in IRAK-1-immunoprecipitates of postnuclear supernatants after 5 and 120 min of IL-1-stimulation (Fig. 6, right part). Therefore, phosphorylation of p24 can be achieved with extracts from the cytoplasmic fraction and from nuclei as well. p24 is associated to IRAK-1 but not to the IL1-RI and its phosphorylation strongly depends on a functional IRAK-1 kinase activity either in the cytoplasm or in the nucleus.

Discussion

We here show that nuclear localization of IRAK-1 requires intact kinase activity. Up to now, the role of the kinase activity of IRAK-1 in the IL-1 signaling pathway has remained unclear. Cells lacking IRAK-1 failed to activate NF-κB in response to IL-1 and IRAK-1-deficient mice exhibit an impaired early cytokine responsiveness to parenterally administered IL-1β [37]. In contrast, the kinase-inactive mutant K239S neither inhibited IL-1-stimulated activation of NF-κB nor the activation of the c-Jun N-terminal kinase (JNK) [9]. Also a second kinase-defective IRAK-1 mutant (D340N) was able to activate NF-κB-dependent transcription [11]. Only abrogation of the kinase activity of both IRAK-1 *and* IRAK-4 abolished NF-κB-dependent reporter gene expression, activation of NF-κB and JNK, and endogenous IL-8 gene expression [38]. Therefore, the kinase activities of IRAK-1 and IRAK-4 appear to be redundant for IL-1-mediated signaling.

IRAK-1 was also identified as conserved component in signaling cascades activated by interleukin-18 [39], TNFα [40], and p75-neurotrophin [41]. Toll-like receptors (TLRs) of which 11 members have been cloned so

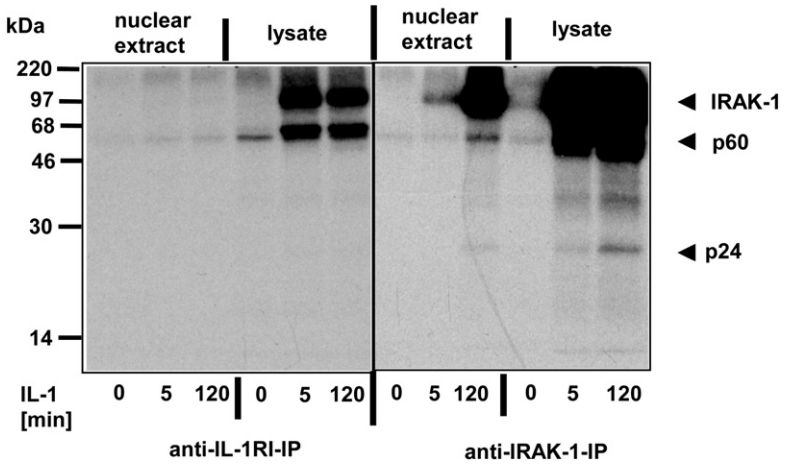

Fig. 6. p24 is associated to IRAK-1 and not to the IL-1RI. EL-4^{IRAK-1} cells were stimulated with IL-1β for the indicated times. Proteins were precipitated from postnuclear supernatants of Brij97 lysates (lysate) or from 150 μg nuclear extract with an anti-IL-1RI- or an anti-IRAK-1-ab and applied to an in vitro kinase assay. Phosphorylated IRAK-1 and the endogenous substrates p24 and p60 were separated onto 12.5% SDS–gels and are marked with arrowheads. Autoradiography exposition time was the same (4 h) for both types of immunoprecipitation. Molecular masses are indicated in kDa on the left.

far have recently emerged to trigger innate and adaptive immune reactions (reviewed in [42]). They recognize distinct pathogen-associated molecular patterns (PAMPs) like bacterial endotoxin LPS (TLR4), bacterial lipoproteins (TLR2), flagellin (TLR5), unmethylated CpG DNA of bacteria and viruses (TLR9), single-stranded viral RNA (TLR7), and double-stranded RNA (TLR3). TLRs also use IRAK-1 for activation of the IKK complex or mitogen-activated protein kinases which leads to a NF-κB- or AP-1-dependent transcriptional response. Hence, inhibition of IRAK-1 translocation into the nucleus might be a general mechanism to interfere with different inflammatory signaling cascades.

Various kinases require an enzymatically active kinase domain to enable their nuclear translocation, e.g., calcium/calmodulin-dependent protein kinase IV [43] or the cyclin-dependent kinase Cdk9 which has to be autophosphorylated for nuclear translocation [44]. Phosphorylation of MAP kinase by the upstream kinase MEK is a prerequisite for its nuclear entry [45]. Also stress-induced nuclear export of kinases such as MAPKAP kinase 2 is regulated by phosphorylation: T334 is herein located between the kinase domain and the C-terminal regulatory domain and acts as a switch for nuclear import and export [46,47]. As demonstrated here IRAK-1 has to be recruited to the IL-1RI, where it becomes highly phosphorylated to subsequently translocate into the nucleus. Kinase-defective IRAK-1 does not translocate. IRAK-1 is active in nuclear extracts and phosphorylates exogenous substrates like histone H1 and myelin basic protein when assayed 'ex vivo' and becomes autophosphorylated. It cannot be ruled out from these assays that this phosphorylation might be achieved by a co-precipitated kinase. This is, nevertheless, unlikely since the nuclear extracts were prepared under high salt (400 mM) conditions and the amount of co-precipitated proteins in an IRAK-1-immunoprecipitate should, therefore, be low if present at all.

So far, only tollip [48,49] and pellino-2 [50] have been described as possible physiological substrates for IRAK-1. Recently Huang et al. [2] reported on the phosphorylation of Stat3 by IRAK-1 in the nucleus of peripheral blood mononuclear cells of atherosclerotic patients with elevated serum IL-10 levels. It could indeed be demonstrated by chromatin immunoprecipitation assays that IRAK-1 binds to the endogenous IL-10 promoter region in THP-1 cells upon lipopolysaccharide stimulation [2]. This indicates that IRAK-1 may have still unidentified targets in the nucleus and that it is not restricted to the IL-1 signaling cascade (see above). Thus, prevention of IRAK-1 recruitment to the IL-1RI, its phosphorylation [32,35], and subsequent translocation into the nucleus by thiol modifying agents like menadione would also suppress a putative gene regulatory function of IRAK-1. Here we show that the phosphorylation of an additional endogenous substrate with an apparent molecular weight of 24 kDa correlates with enzymatic activity and phosphorylation of nuclear located IRAK-1. Phosphorylation of p24 is not detectable in the kinase negative mutants suggesting that it is a specific target of IRAK-1. These results were confirmed by experiments showing that p24 associates to IRAK-1, but not to the IL-1RI. Phosphorylation of p24 is detectable in the cytoplasm upon short IL-1 stimulation and in the nucleus after prolonged times of IL-1 stimulation which enabled IRAK-1 translocation. Kinase-active IRAK-1 and p24 as its substrate might therefore exist as well in the cytosol as in the nuclear compartment depending on the IL-1 stimulation time.

In conclusion, our study defines p24 as a novel endogenous substrate for IRAK-1, which is detectable in the cytoplasm and in the nucleus. IRAK-1 can act as a kinase in the nuclear compartment and is able to autophosphorylate and to phosphorylate p24 in vitro. Nuclear translocation and, hence, phosphorylation of p24 is not observed in kinase-negative mutants which demonstrates that intracellular trafficking of IRAK-1 requires recruitment to the IL-1RI and an intact kinase activity. Further investigation is required to identify the nature of p24.

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References

[1] G. Böl, O.J. Kreuzer, R. Brigelius-Flohé, Translocation of the interleukin-1 receptor-associated kinase-1 (IRAK-1) into the nucleus, FEBS Lett. 477 (2000) 73–78.
[2] Y. Huang, T. Li, D.C. Sane, L. Li, IRAK1 serves as a novel regulator essential for lipopolysaccharide-induced interleukin-10 gene expression, J. Biol. Chem. 279 (2004) 51697–51703.
[3] M.U. Martin, H. Wesche, Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family, Biochim. Biophys. Acta 1592 (2002) 265–280.
[4] C. Kollewe, A.C. Mackensen, D. Neumann, J. Knop, P. Cao, S. Li, H. Wesche, M.U. Martin, Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling, J. Biol. Chem. 279 (2004) 5227–5236.
[5] M. Martin, G.F. Böl, A. Eriksson, K. Resch, R. Brigelius-Flohé, Interleukin-1-induced activation of a protein kinase co-precipitating with the type I interleukin-1 receptor in T cells, Eur. J. Immunol. 24 (1994) 1566–1571.
[6] Z. Cao, W.J. Henzel, X. Gao, IRAK: a kinase associated with the interleukin-1 receptor, Science 271 (1996) 1128–1131.
[7] H. Wesche, X. Gao, X. Li, C.J. Kirschning, G.R. Stark, Z. Cao, IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family, J. Biol. Chem. 274 (1999) 19403–19410.
[8] M. Muzio, J. Ni, P. Feng, V.M. Dixit, IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling, Science 278 (1997) 1612–1615.
[9] J. Knop, M.U. Martin, Effects of IL-1 receptor-associated kinase (IRAK) expression on IL-1 signaling are independent of its kinase activity, FEBS Lett. 448 (1999) 81–85.
[10] X. Li, M. Commane, C. Burns, K. Vithalani, Z. Cao, G.R. Stark, Mutant cells that do not respond to interleukin-1 (IL-1) reveal a novel role for IL-1 receptor-associated kinase, Mol. Cell. Biol. 19 (1999) 4643–4652.
[11] B. Maschera, K. Ray, K. Burns, F. Volpe, Overexpression of an enzymically inactive interleukin-1-receptor-associated kinase activates nuclear factor-kappaB, Biochem. J. 339 (1999) 227–231.
[12] X. Li, M. Commane, Z. Jiang, G.R. Stark, IL-1-induced NFkappa B and c-Jun N-terminal kinase (JNK) activation diverge at IL-1 receptor-associated kinase (IRAK), Proc. Natl. Acad. Sci. USA 98 (2001) 4461–4465.
[13] K. Ross, L. Yang, S. Dower, F. Volpe, F. Guesdon, Identification of threonine66 as a functionally critical residue of the interleukin-1 receptor-associated kinase, J. Biol. Chem. 277 (2002) 37414–37421.
[14] E.L. Cooke, I.J. Uings, C.L. Xia, P. Woo, K.P. Ray, Functional analysis of the interleukin-1-receptor-associated kinase (IRAK-1) in interleukin-1 beta-stimulated nuclear factor kappa B (NF-kappa B) pathway activation: IRAK-1 associates with the NF-kappa B essential modulator (NEMO) upon receptor stimulation, Biochem. J. 359 (2001) 403–410.
[15] B.M. Curtis, M.B. Widmer, P. deRoos, E.E. Qwarnstrom, IL-1 and its receptor are translocated to the nucleus, J. Immunol. 144 (1990) 1295–1303.
[16] S. Grenfell, N. Smithers, K. Miller, R. Solari, Receptor-mediated endocytosis and nuclear transport of human interleukin1 alpha, Biochem. J. 264 (1989) 813–822.
[17] S. Grenfell, N. Smithers, S. Witham, A. Shaw, P. Graber, R. Solari, Analysis of mutations in the putative nuclear localization sequence of interleukin-1 beta, Biochem. J. 280 (1991) 111–116.
[18] A. Heguy, C. Baldari, K. Bush, R. Nagele, R.C. Newton, R.J. Robb, R. Horuk, J.L. Telford, M. Melli, Internalization and nuclear localization of interleukin1 are not sufficient for function, Cell Growth Differ. 2 (1991) 311–315.
[19] K. Kuno, S. Okamoto, K. Hirose, S. Murakami, K. Matsushima, Structure and function of the intracellular portion of the mouse interleukin 1 receptor (type I). Determining the essential region for transducing signals to activate the interleukin 8 gene, J. Biol. Chem. 268 (1993) 13510–13518.
[20] J.A. Maier, M. Statuto, G. Ragnotti, Endogenous interleukin 1 alpha must be transported to the nucleus to exert its activity in human endothelial cells, Mol. Cell. Biol. 14 (1994) 1845–1851.
[21] G.A. McMahon, S. Garfinkel, I. Prudovsky, X. Hu, T. Maciag, Intracellular precursor interleukin (IL)-1alpha, but not mature IL-1alpha, is able to regulate human endothelial cell migration in vitro, J. Biol. Chem. 272 (1997) 28202–28205.
[22] R. Solari, N. Smithers, N. Kennard, K. Ray, S. Grenfell, Receptor mediated endocytosis and intracellular fate of interleukin 1, Biochem. Pharmacol. 47 (1994) 93–101.
[23] M.N. Weitzmann, N. Savage, Nuclear internalisation and DNA binding activities of interleukin-1, interleukin-1 receptor and interleukin-1/receptor complexes, Biochem. Biophys. Res. Commun. 187 (1992) 1166–1171.
[24] A. Bavelloni, S. Santi, A. Sirri, M. Riccio, I. Faenza, N. Zini, S. Cecchi, A. Ferri, P. Auron, N.M. Maraldi, S. Marmiroli, Phosphatidylinositol 3-kinase translocation to the nucleus is induced by interleukin 1 and prevented by mutation of interleukin 1 receptor in human osteosarcoma Saos-2 cells, J. Cell Sci. 112 (1999) 631–640.
[25] G. Sacht, R. Brigelius-Flohé, M. Kiess, H. Sztajer, L. Flohé, ATP-sensitive association of mortalin with the IL-1 receptor type I, Biofactors 9 (1999) 49–60.
[26] A. Heguy, C.T. Baldari, G. Macchia, J.L. Telford, M. Melli, Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the *Drosophila* toll protein are essential for IL-1R signal transduction, J. Biol. Chem. 267 (1992) 2605–2609.
[27] J.H. Wessendorf, S. Garfinkel, X. Zhan, S. Brown, T. Maciag, Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor, J. Biol. Chem. 268 (1993) 22100–22104.
[28] R. Brigelius-Flohé, A. Banning, M. Kny, G.F. Böl, Redox events in interleukin-1 signaling, Arch. Biochem. Biophys. 423 (2004) 66–73.
[29] B. Meier, H.H. Radeke, S. Selle, M. Younes, H. Sies, K. Resch, G.G. Habermehl, Human fibroblasts release reactive oxygen

species in response to interleukin-1 or tumour necrosis factor-alpha, Biochem. J. 263 (1989) 539–545.
[30] M. Sundaresan, Z.X. Yu, V.J. Ferrans, D.J. Sulciner, J.S. Gutkind, K. Irani, P.J. Goldschmidt-Clermont, T. Finkel, Regulation of reactive-oxygen-species generation in fibroblasts by Rac1, Biochem. J. 318 (1996) 379–382.
[31] G. Bonizzi, J. Piette, S. Schoonbroodt, R. Greimers, L. Havard, M.P. Merville, V. Bours, Reactive oxygen intermediate-dependent NF-kappaB activation by interleukin-1beta requires 5-lipoxygenase or NADPH oxidase activity, Mol. Cell. Biol. 19 (1999) 1950–1960.
[32] F. Tewes, G.F. Böl, R. Brigelius-Flohé, Thiol modulation inhibits the interleukin (IL)-1-mediated activation of an IL-1 receptor-associated protein kinase and NF-kappa B, Eur. J. Immunol. 27 (1997) 3015–3021.
[33] G.-F. Böl, F. Tewes, R. Brigelius-Flohé, Interleukin-1 signaling is dependent on free thiols, Biofactors 10 (1999) 175–178.
[34] B. Friedrichs, C. Müller, R. Brigelius-Flohé, Inhibition of tumor necrosis factor-alpha- and interleukin-1-induced endothelial E-selectin expression by thiol-modifying agents, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 1829–1837.
[35] G.F. Böl, N. Jurrmann, R. Brigelius-Flohé, Recruitment of the interleukin-1 receptor (IL-1RI)-associated kinase IRAK to the IL-1RI is redox regulated, Biol. Chem. 384 (2003) 609–617.
[36] N. Jurrmann, R. Brigelius-Flohé, G.-F. Böl, Curcumin blocks interleukin-1 (IL-1) signaling by inhibiting the recruitment of the IL-1 receptor-associated kinase IRAK in murine thymoma EL-4 cells, J. Nutr., 2005, in revision.
[37] J.A. Thomas, J.L. Allen, M. Tsen, T. Dubnicoff, J. Danao, X.C. Liao, Z. Cao, S.A. Wasserman, Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase, J. Immunol. 163 (1999) 978–984.
[38] J. Qin, Z. Jiang, Y. Qian, J.L. Casanova, X. Li, IRAK4 kinase activity is redundant for IRAK phosphorylation and IL-1 responsiveness, J. Biol. Chem. 279 (2004) 26748–26753.
[39] H. Kojima, M. Takeuchi, T. Ohta, Y. Nishida, N. Arai, M. Ikeda, H. Ikegami, M. Kurimoto, Interleukin-18 activates the IRAK–TRAF6 pathway in mouse EL-4 cells, Biochem. Biophys. Res. Commun. 244 (1998) 183–186.
[40] E. Vig, M. Green, Y. Liu, D.B. Donner, N. Mukaida, M.G. Goebl, M.A. Harrington, Modulation of tumor necrosis factor and interleukin-1-dependent NF-kappaB activity by mPLK/IRAK, J. Biol. Chem. 274 (1999) 13077–13084.
[41] V. Mamidipudi, X. Li, M.W. Wooten, Identification of interleukin 1 receptor-associated kinase as a conserved component in the p75-neurotrophin receptor activation of nuclear factor-kappa B, J. Biol. Chem. 277 (2002) 28010–28018.
[42] A. Iwasaki, R. Medzhitov, Toll-like receptor control of the adaptive immune responses, Nat. Immunol. 5 (2004) 987–995.
[43] S.M. Lemrow, K.A. Anderson, J.D. Joseph, T.J. Ribar, P.K. Noeldner, A.R. Means, Catalytic activity is required for calcium/calmodulin-dependent protein kinase IV to enter the nucleus, J. Biol. Chem. 279 (2004) 11664–11671.
[44] G. Napolitano, B. Majello, L. Lania, Catalytic activity of Cdk9 is required for nuclear co-localization of the Cdk9/cyclin T1 (P-TEFb) complex, J. Cell. Physiol. 197 (2003) 1–7.
[45] M. Fukuda, Y. Gotoh, E. Nishida, Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase, EMBO J. 16 (1997) 1901–1908.
[46] W. Meng, L.L. Swenson, M.J. Fitzgibbon, K. Hayakawa, E. Ter Haar, A.E. Behrens, J.R. Fulghum, J.A. Lippke, Structure of mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 suggests a bifunctional switch that couples kinase activation with nuclear export, J. Biol. Chem. 277 (2002) 37401–37405.
[47] K. Engel, A. Kotlyarov, M. Gaestel, Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation, EMBO J. 17 (1998) 3363–3371.
[48] K. Burns, J. Clatworthy, L. Martin, F. Martinon, C. Plumpton, B. Maschera, A. Lewis, K. Ray, J. Tschopp, F. Volpe, Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor, Nat. Cell Biol. 2 (2000) 346–351.
[49] G. Zhang, S. Ghosh, Negative regulation of toll-like receptor-mediated signaling by Tollip, J. Biol. Chem. 277 (2002) 7059–7065.
[50] A. Strelow, C. Kollewe, H. Wesche, Characterization of Pellino 2, a substrate of IRAK1 and IRAK4, FEBS Lett. 547 (2003) 157–161.