Translocation of the interleukin-1 receptor-associated kinase-1 (IRAK-1) into the nucleus

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Received 23 May 2000

Edited by Marco Baggiolini

Abstract Interleukin-1 (IL-1) signal transduction involves the recruitment of the IL-1 receptor-associated kinase-1 (IRAK-1). Subsequent signaling finally leads to nuclear translocation of NFκB. We here show that the association and autophosphorylation of IRAK-1 was already detectable 30 s after IL-1 stimulation of ECV 304 cells. Significant levels of IRAK-1 accumulated in the nucleus 30 min after IL-1 stimulation shown by Western blot analysis and confocal laser scanning microscopy. Nuclear transfer of IRAK-1 upon IL-1 stimulation was confirmed in the murine T cell line EL-4. This characterizes nuclear localization of IRAK-1 as a possibly essential event in the IL-1 signaling cascade.

Key words: Interleukin-1; Interleukin-1 receptor type I; Interleukin-1 receptor-associated kinase-1; Translocation; Nucleus

1. Introduction

For long it remained an enigma how interleukin-1 (IL-1) binding to its receptor at the cellular surface leads to gene activation in the nucleus. The first step in unravelling IL-1 signaling was the discovery of a serine/threonine kinase that becomes associated with the IL-1 receptor type I (IL-1R I) upon IL-1 binding [1]. In the meantime, this IL-1 receptor-associated kinase-1 (IRAK) has been cloned [2] and renamed IRAK-1 after further kinases were found to bind to IL-1 receptors in various cells [3,4]. By now, the following key events were reported to contribute to the formation of the IL-1RI signaling complex: response to IL-1 depends on the presence of the IL-1R accessory protein (IL-1R-AcP) [5,6] which interacts with the IL-1RI [7,8] and also with IRAK-1 [8,9]; IRAK-1 binding to IL-1RI and to IL-1R-AcP is supported by the adapter protein MyD88 [10,11]; within the receptor complex IRAK-1 is autophosphorylated [2,12] and also phosphorylates an endogenous substrate pp60 [1]. Downstream signaling by the activated IL-1 receptor complex is mediated by a member of the TNF-associated factor family, TRAF6 [13], and involves the activation of the TGF-β-activated kinase (TAK) [14], NFκB-inducing kinase (NIK) [15] and IκB kinase (IKK) [16]. The latter enables activation of NFκB and its translocation into the nucleus which appears to be an essential consequence of cellular activation by IL-1. Beyond this main signaling route cross-talk to MAP kinases [17–19] and phosphatidyl-inositol-3 (PI3) kinase [20–22] pathways have been demonstrated.

The emerging view of IL-1-triggered phosphorylation cascades is complemented by observations indicating receptor-mediated trafficking of IL-1 to the nucleus. Both, IL-1 [23–27] and the IL-1RI [26,28], as well as the receptor-associated PI3-kinase [29] 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 [25,26]. The potential relevance of IL-1 trafficking is further supported by (i) the detection of two proteins with nucleic acid binding sequence characteristics in the immune-precipitated IL-1RI complex [30], (ii) the association of the chaperone-type HSP74 (Mortalin) [30] with the IL-1 receptor, and (iii) putative nuclear localization sequences (NLS) in the cytoplasmic domain of the IL-1RI [26,31], in the PI3-kinase [29], in mature IL-1β [24] and in the precursor but not the mature form of IL-1α [32,33]. It is not clear, however, whether the nuclear localization of IL-1RI and IL-1β are essential for signaling [26,34] whereas it regulates IL-1α precursor-mediated endothelial cell functions [33,35].

We thus looked whether other components of the IL-1RI complex might undergo cellular trafficking. We found that IRAK-1 was translocated into the nucleus upon IL-1 stimulation of ECV 304 and EL-4 cells. It there appeared synchronously with activated NFκB lending support to the idea of a potential synergy of NFκB activation and receptor complex trafficking.

2. Materials and methods

2.1. Cell culture and stimulation

ECV 304, described as a human umbilical cord vein endothelial cell line, was recently reported to be rather a derivative of the human epithelial urinary bladder line T24 [36]. Despite this uncertainty, the cell line remained in this study because the cellular response to IL-1 investigated was not considered specific for endothelial cells. ECV 304 cells were grown in medium M199 EARLE supplemented with 5% FCS, 0.68 mM L-glutamine, 0.22% $NaHCO_3$, 5 mM HEPES, 50 U/ml penicillin–streptomycin. EL-4 cells were cultured in RPMI 1640, 2 mM L-glutamine and 5% FCS. Both cell lines were washed two times with medium lacking FCS and then treated with or without 10 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.

2.2. In vitro kinase assay

After stimulation with IL-1, cells were washed three times with PBS and lysed in 1% Brij98 (Sigma), 50 mM NaCl, 50 mM Tris, pH 7.4 at 4°C for 1 h. Postnuclear supernatants of 2×10^7 cells were incubated with 1 μg anti-IL-1-RI-antibody (Pharmingen) and 50 μl of a protein G-Sepharose slurry at 4°C overnight. Immunoprecipitates were

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

0014-5793/00/$20.00
PII: S0014-5793(00)01759-2

washed three times in lysis buffer and three times in kinase buffer consisting of 100 mM NaCl, 20 mM HEPES, 5 mM $MnCl_2$, 5 mM $MgCl_2$, pH 6.5. The in vitro kinase assay was performed by adding 2 μCi ^{32}P-γ-ATP for 10 min at room temperature. The kinase reaction was stopped with kinase buffer containing 20 mM EDTA, samples washed five times with the same buffer and proteins separated from antibody and protein G-Sepharose by boiling for 5 min in Laemmli buffer. Sepharose was removed by centrifugation and proteins separated on 5–7.5% SDS–PAGE gradient gels. Dried gels were autoradiographed on Kodak X-Omat films.

2.3. Nuclear extracts

ECV and EL-4 cells were washed in PBS and resuspended in PBS containing 5 mM $MgCl_2$, 1 mM PMSF, 1 mM benzamidine–HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin and the protease-inhibitor-cocktail complete™ (Roche, Mannheim, Germany). Pottered cells were centrifuged for 10 min at 600×*g*. Pellets containing intact nuclei were washed in PBS with protease inhibitors, resuspended in 20 mM Tris, pH 7.5, 250 mM sucrose, 10 mM $MgCl_2$, and further purified by ultracentrifugation (80 000×*g*, 1 h) through 30% sucrose in the same buffer. Pellets were washed three times with PBS and nuclear protein was extracted with high salt buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). IRAK-1 was precipitated from 50 μg nuclear protein by incubation with 0.5 μl anti-IRAK-1-antiserum (C-terminal, aa 699–712, Calbiochem) and 50 μl of a protein G-Sepharose slurry at 4°C overnight. Samples were washed six times with 50 mM NaCl, 50 mM Tris, pH 7.4 and prepared for gel separation as described above. 10% SDS–PAGE gels were immunoblotted to nitrocellulose.

2.4. Western blotting

Western blot analysis was performed with an anti-IRAK-1-antiserum (1:4000, C-terminal, aa 699–712, Calbiochem) or an anti-IRAK-1-antibody (1 μg/ml, aa 440–712, Santa Cruz) as indicated. As second antibody a POD-conjugated goat anti-rabbit antibody (Roche, 1:3000) was used. Bands were made visible by chemiluminescence imaging with the Fuji LAS1000-CCD-camera system.

2.5. Immunofluorescence

ECV 304 cells were seeded for adherence in 24 well plates overnight. PBS washed cells were permeabilized with ice cold acetone/methanol (1+1, 2 min), washed, blocked with 2% swine serum and incubated overnight with anti-IRAK-1-antibody (C-terminal, aa 440–712, 400 ng/ml, Santa Cruz). After washing, TRITC-conjugated swine anti-rabbit antibody (1:100, DAKO) was added for 1 h at room temperature. Hoechst 33258 (1 μg/ml) was added for 30 min to visualize cell nuclei. Washed cells were embedded in glycerol–gelatine and analyzed using a Zeiss Laser Scan Inverted 410 Axiovert microscope with an argon–krypton laser. Optical sections were taken every 0.2 μm (512×512 pixel images).

3. Results

3.1. Transient recruitment of IRAK-1 to the IL-1RI

Already 30 s after stimulation of ECV 304 cells with IL-1, IRAK-1 was associated with the IL-1RI as observed by co-precipitation with the IL-1RI antibody and immunostaining with an IRAK-1 antibody (Fig. 1A). In the absence of IL-1 no IRAK-1 was found associated with the receptor. IRAK-1 migrated at a molecular weight of about 80 kDa (lower band) and as a diffuse band at a molecular range of 85–110 kDa which is characteristic for the phosphorylated form. The maximum amount was coprecipitated after 1 min, then IRAK-1 disappeared and was hardly detectable after 15 min of IL-1 stimulation.

3.2. Transient phosphorylation of IRAK-1 associated to the IL-1RI

IL-1-induced phosphorylation of IRAK-1 in ECV 304 cells was estimated by an in vitro kinase assay in immunoprecipitates of postnuclear supernatants (Fig. 1B). While background phosphorylation in untreated cells was barely detectable (Fig. 1B, lane 1), phosphorylation of IRAK-1 became detectable 30 s after IL-1 stimulation as a diffuse band with an apparent molecular weight of about 80–100 kDa (Fig. 1B, lane 2) and disappeared after 15 min. Phosphorylation of IRAK-1 thus showed the same kinetics as its recruitment to the receptor indicating that once bound to the receptor, IRAK-1 becomes phosphorylated either via autophosphorylation or by another associated kinase. The latter idea is supported by the fact that phosphorylation of p60 did not change when IRAK-1 was released from the receptor (Fig. 1B).

3.3. Detection of IRAK-1 in nuclear protein extracts by Western blot analysis

60 min after IL-1 stimulation association of IRAK-1 to the receptor was neither detectable by the in vitro kinase assay nor by Western blot analysis (not shown). Also, no IRAK-1 was detected in postnuclear supernatants (not shown). This indicates either degradation or changed subcellular distribution of IRAK-1. We, therefore, investigated whether it could have been transported into the nucleus in ECV 304 cells and,

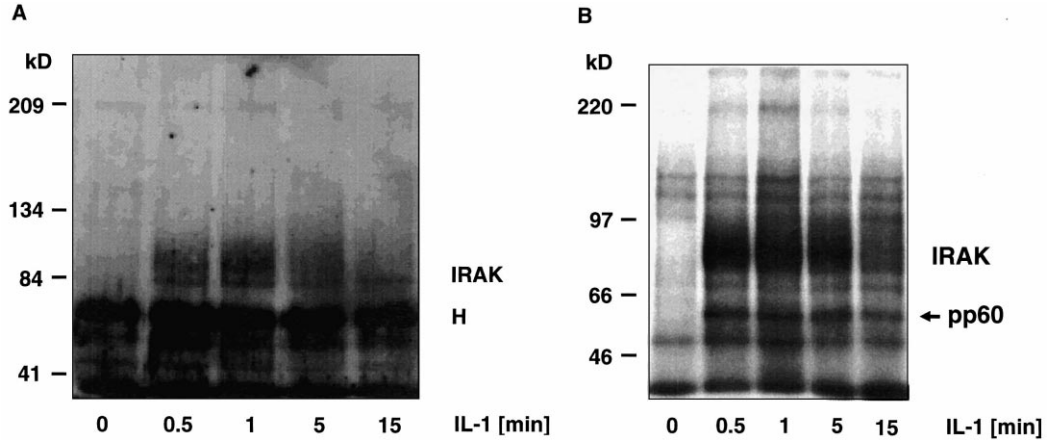

Fig. 1. Transient coprecipitation of IRAK-1 with the IL-1-RI in ECV 304 cells. Cells were stimulated with IL-1 for the times indicated. Supernatants of Brij98 lysates were immunoprecipitated with an anti-IL-1-RI-antibody. A: Washed samples were loaded onto a 5–7.5% SDS–PAGE gradient gel. Western blot detection was performed with an IRAK-1-antibody (1 μg/ml, Santa Cruz) and a POD-conjugated goat anti-rabbit antibody (Roche, 1:3000). H indicates the heavy chain of the antibody used for immunoprecipitation. B: Immunoprecipitates were used for the in vitro kinase assay described in Section 2. Shown is an autoradiograph of a 5–7.5% SDS–PAGE gradient gel.

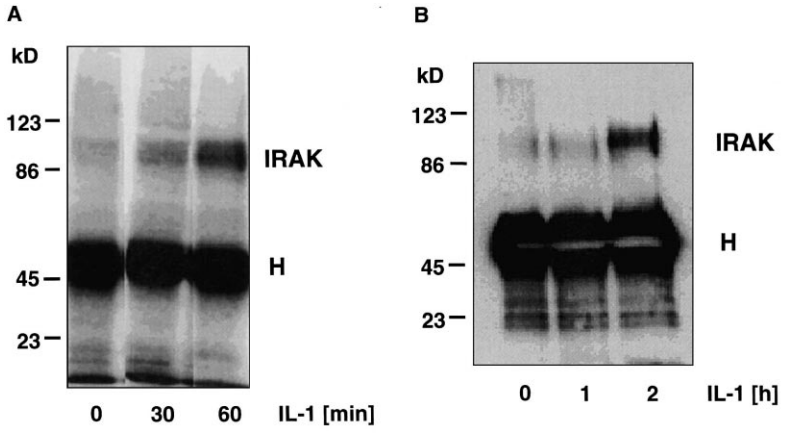

Fig. 2. Immunostaining of IRAK-1 in nuclear extracts of IL-1 stimulated cells. ECV 304 (A) and EL-4 (B) cells were preincubated with or without IL-1β (10 ng/ml) for the times indicated. IRAK-1 was precipitated from 50 μg nuclear extracts with an anti-IRAK-1-antiserum (C-terminal, aa 699–712, Calbiochem), the precipitate separated on a 10% SDS gel and immunoblotted to nitrocellulose membranes. IRAK-1 was made visible with a serum dilution of 1:4000, a POD-conjugated second antibody and the Fuji LAS1000-CCD-camera system. H marks the unspecific reaction of the heavy chain of the antiserum. For further experimental details see Section 2.

in addition, in EL-4 cells. Purity of nuclear extracts was controlled by measurement of the marker enzymes lactate dehydrogenase, glucose-6-phosphate-dehydrogenase, phosphoglucose isomerase (cytosol), cytochrome *c*-oxidase (mitochondria), alkaline phosphatase (membranes) and by estimation of the DNA content at 600 nm following Dische et al. [37]. All enzymes were found to be less than 8% in nuclear extracts compared to their native localization sites.

IL-1-stimulated phosphorylation was not observed in in vitro kinase assays with IRAK-1 precipitated from nuclear extracts (not shown). In contrast, IRAK-1 protein, which was negligible in nuclear extracts of control cells (Fig. 2A,B, lane 1), increased significantly following 30 and 60 min of IL-1 stimulation in ECV 304 cells or following 1 and 2 h in EL-4 cells (Fig. 2A,B, lanes 2 and 3), respectively.

3.4. Detection of IRAK-1 in the nucleus by laser scanning microscopy

To verify the Western blot data of nuclear located IRAK-1 we performed laser scanning microscopy. IRAK-1 was located in the cytosol of unstimulated ECV 304 cells (Fig. 3A, left part). Following 30 min of IL-1 stimulation IRAK-1 was detected at the nuclear membrane and as a bulk being transported through the nuclear membrane into the nucleus (Fig. 3A, middle part). After 1 h of IL-1 stimulation the amount of IRAK-1 in the cytosol was significantly diminished and most of IRAK-1 was located in the nucleus in a condensed form (Fig. 3A, right part). Cells which were incubated with the second antibody only showed unspecific staining not significant for IRAK-1 (Fig. 3A, insert).

3.5. Nuclear localization of IRAK-1 occurs simultaneously with NFκB

Translocation of NFκB was monitored during the same time scale of IL-1 stimulation (Fig. 3B). Prior to IL-1, NFκB was only present in the cytosol (Fig. 3B, left part). 30 min after IL-1 stimulation most of the NFκB protein could be localized at the nuclear membrane and in the nucleus of ECV 304 cells (Fig. 3B, middle part). After 1 h of IL-1 stimulation NFκB was significantly decreased in the cytosol, instead it was found in a condensed form in the nucleus as was observed for IRAK-1 (Fig. 3B, right part).

4. Discussion

We here demonstrate the accumulation of IRAK-1 in the nucleus in response to IL-1. The data, obtained by two different methods and with two different cell lines indicate that translocation of IRAK-1 might be an essential part in the signaling cascade of IL-1. Nuclear translocation of IL-1RI-associated proteins so far has not been studied in detail and only a few data on IL-1 and IL-1RI translocation are available. Internalization of the IL-1 receptor upon IL-1 binding was first shown in 1987 [38]. Whereas the receptor was found to be downregulated, IL-1 was not degraded after internalization but rather accumulated in the nuclei of EL-4 cells 2–4 h after stimulation suggesting a so far unexplained function of IL-1 in the nucleus [38]. Although the IL-1-loaded IL-1 receptor was shown to unspecifically bind to DNA [28], a postulated nuclear IL-1 receptor has never been identified. It is even controversially discussed whether internalization and nuclear localization of IL-1 are required to trigger signals at all [25–27]. It might thus not be IL-1 itself but other proteins from the IL-1RI complex which must be transported into the nucleus for function.

The recent finding that upon stimulation with IL-1 PI3-kinase is first recruited to the IL-1RI [20,21] or AcP [22] and then transported into the nucleus [29] demonstrates (i) that multiple kinases act in the IL-1RI complex and (ii) that cellular trafficking might be equally involved. Translocation of PI3-kinase into the nucleus occurred within 15 min and was transient [29]. It could be prevented by a point mutation in the IL-1RI binding site (Y479F) for the p85 subunit of PI3-kinase and by PI3-kinase inhibitors [29]. Thus binding to the IL-1RI as well as kinase activity is required for nuclear translocalization of PI3-kinase. Whether the kinase activity of IRAK-1 is similarly required for nuclear localization is not known. Interestingly, HEK-293 cells overexpressing a kinase-defective mutant of IRAK-1 (D340N) were still able to activate NFκB following IL-1 stimulation [39]. Overexpression of

a second kinase-inactive IRAK-1 mutant (K239S) in EL-4 cells did not inhibit IL-1-induced activation of c-Jun N-terminal kinase, NFκB and IL-2 production [40]. It was therefore suggested that the kinase activity of IRAK-1 is not required for downstream signaling at all. That we did not find any IRAK-1 activity in the nuclei of IL-1-stimulated cells (not shown) might support this idea.

Nuclear localization of proteins larger than 45 kDa requires sorting sequences, called NLS. A protein, called importin, directly binds to such sequences, thereby driving transport of the respective protein into the nucleus [41]. Such transport is energy-dependent and utilizes the GDP-bound small GTPase Ran [42]. A nuclear localization signal is present in the human IRAK-1 at aa 503–508 (RRXKRR) but mouse IRAK-1 lacks such a signal. Thus, IRAK-1 most probably is not transported as such, it is rather supported by other sorting proteins. Possible candidates might be the receptor, as suggested for the transport for IL-1, or mortalin, the chap-

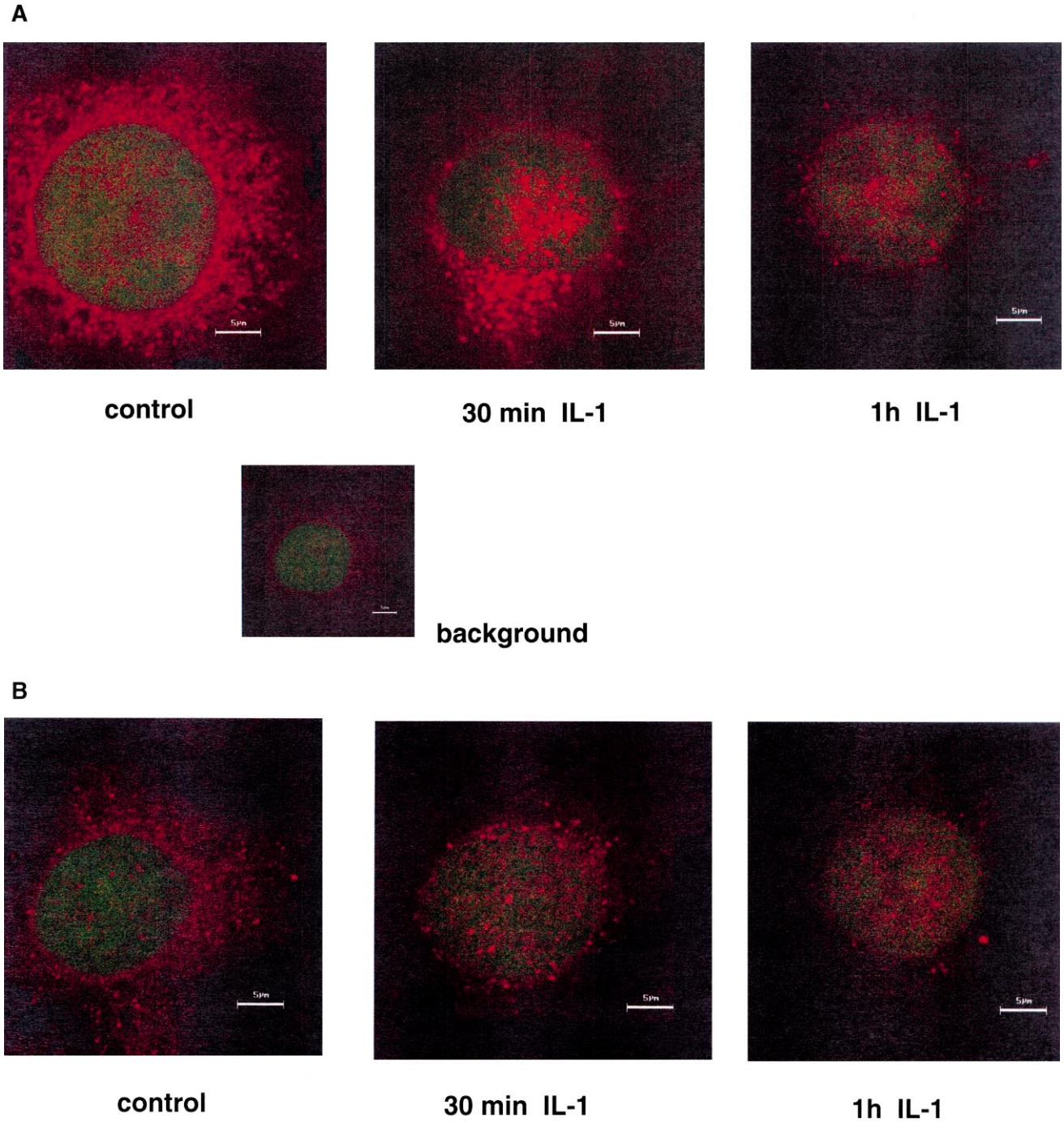

Fig. 3. Subcellular distribution of IRAK-1 and NFκB in IL-1-stimulated ECV 304 cells. ECV 304 cells were preincubated with or without IL-1β (10 ng/ml) for the times indicated. Permeabilized cells were incubated with an anti-IRAK-1-antibody (A), or an anti-NFκBp65-antibody (B) and followed by a TRITC-conjugated swine anti-rabbit-antibody. Hoechst 33258 was used to visualize cell nuclei. Cells were analyzed using a Zeiss Laser Scan Inverted 410 Axiovert microscope with an argon–krypton laser. Sections through the center of the cells are shown (512×512 pixel images). Red color shows fluorescence of IRAK-1, green fluorescence shows nuclei (changed from blue). Bars indicate 5 μm.

erone-type heat shock protein 74, which has been found to be associated with the IL-1RI complex [30]. A typical nuclear import sequence, KXRRR is present in IL-1R and IL-1β [24] and deletion of such nuclear localization signals in the IL-1RI prevented the induction of IL-2 by IL-1 [25] in human fibroblasts, but did not affect IL-1-mediated IL-8 induction in the human Jurkat T cell line transfected with the mouse IL-1RI [34]. Since the amino acids identified as essential for signal transduction are different in human and mouse IL-1RI (aa 477–527 for human [25] versus aa 364–474 for mouse [34] IL-1RI) the failure to block IL-8 gene activation might be due to an impairment of the interactions of the murine IL-1RI with human components of the IL-1RI signaling complex. The essential region in the IL-1RI now is known as the so-called TIR domain (Toll/IL-1-receptor domain) [31]. There MyD88 binds with its own TIR domain thereby presenting the death domain for the interaction with IRAK-1 [11]. Thus, the deletion of diverse NLSs equally resulted in the deletion of the interaction site for IRAK-1 which might explain the observed effects.

IRAK-1 is relevant to NFκB. Mutations in the cytoplasmic domain of IL-1RI (aa 508–521) prevented coprecipitation of IRAK-1 and IL-1RI and blocked NFκB activation [43]. A concurrent loss of IRAK-1 and NFκB activation was observed by deletion of a C-terminal part in the cytoplasmic domain of IL-1RI, called box 3 spanning aa 467–515 [44]. Finally, cells made deficient in IRAK-1 by gene targeting are no longer able to activate NFκB in response to IL-1 [45], whereas the response to TNF remained. In summary, genetic manipulations affecting IRAK-1 association to the receptor affect NFκB activation while kinase activity does not. The mechanism linking receptor association and transcellular trafficking of IRAK-1 to NFκB activation is far from being understood. In a recent study [12] a transient recruitment of IRAK-1 to the IL-1RI in MRC-5 lung fibroblasts was shown. The disappearance of IRAK-1 from the receptor correlated with the loss of IκB and could be delayed but not abolished by proteasome inhibition. This was interpreted as proteasomal degradation of IRAK-1. A redistribution of IRAK-1 into subcellular compartments, however, was not shown. Based on the data presented here, the observed decline of IRAK-1 equally complies with alternative explanations. IRAK-1 translocation to the nucleus (i) could occur in association with NFκB, (ii) it could be supported by components of the NFκB system, or (iii) could facilitate the transport of NFκB itself. An inhibition of IκB degradation would then prevent both translocation of NFκB and of IRAK-1.

The function of nuclear IRAK-1 remains speculative. The questioned relevance of IRAK-1 kinase activity (see above) and the failure to detect any enhanced nuclear kinase activity after import of IRAK-1, however, clearly point to a distinct role of the translocated IRAK-1 in IL-1 signaling that complements the established IL-1-dependent cytosolic cascade of phosphorylations. The pleiotropic effects of IL-1 may require a broad arsenal of signaling pathways comprising the activation of diverse kinases and cellular trafficking of components of the IL-1RI complex that exert their action at the nucleus level by enabling or modulating the efficacy of transcription factors.

Acknowledgements: The excellent technical assistance of G. Aust and E. Wendt is gratefully acknowledged.

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