The Interleukin-1 Receptor-associated Kinase Is Degraded by Proteasomes following Its Phosphorylation*

Ting-Ting Yamin and Douglas K. Miller‡

From the Department of Inflammation Research, Merck Research Laboratories, Rahway, New Jersey 07065

Following interleukin (IL)-1 stimulation, the majority of the cellular interleukin-1 receptor-associated kinase (IRAK) translocates to a discrete subset of the Type I IL-1 receptor (IL-1R1) in MRC-5 human lung fibroblasts. As the IRAK becomes multiphosphorylated, it is degraded by proteasomes at a rate comparable to that of the degradation of the phosphorylated IκBα protein. Proteasome inhibitors block the degradation of phosphorylated IRAK and correspondingly increase the amount of IL-1R1 that can be immunoprecipitated with IRAK. The nonspecific kinase inhibitor K-252b blocks IRAK phosphorylation and degradation, but does not inhibit IRAK association with the IL-1R1 indicating that translocation of IRAK to the IL-1R1 and its phosphorylation are independent events. The IL-1 specificity of these effects is indicated by the lack of IRAK phosphorylation and degradation by IL-1 in the presence of the IL-1 receptor antagonist or by the activation of MRC-5 cells by tumor necrosis factor α. Long term exposure of MRC-5 cells to IL-1 desensitizes the resynthesized IκBα to IL-1, but not to tumor necrosis factor α stimulation, but no additional effects on IRAK are seen.

IL-1 is a master cytokine responsible for the induction of a number of proteins associated with inflammation, such as metallocproteinases, cyclooxygenase, nitric-oxide synthetase, and adhesion proteins (1). Many of these responses are activated by the rapid activation of the transcription factor NF-κB following signal transduction by IL-1α or IL-1β bound to the type I IL-1 receptor (IL-1R1) (see Ref. 2 for review). Activation of the IL-1R1 leads to the activation of at least two kinases resulting in the phosphorylation of IκBα and the activation of NF-κB. Following IL-1 activation, a distinctive Ser/Thr kinase activity binds to and immunoprecipitates with the IL-1R1 (3, 4). This purified IL-1 receptor-associated kinase (IRAK) is highly homologous to the Drosophila kinase Pelle but not to other mammalian Ser/Thr kinases, and it has only a limited ability to phosphorylate IκBα (5, 6). A second Ser/Thr kinase has been identified that is dependent upon ubiquitylation for its subsequent phosphorylation of IκBα (7). Presumably this latter kinase is activated directly by IRAK or indirectly by as yet unidentified intermediate kinases as well as the TNF receptor associated kinases (see Refs. 8–11). The specific phosphorylation of IκBα on Ser-32 and Ser-36 by this ubiquitin-dependent kinase leads to the recognition and destruction of the IκBα by proteasomes, which then frees the NF-κB to translocate into the nucleus and begin transcription (8, 12–16).

A similar signal transduction sequence has been observed in Drosophila. In Drosophila Toll activates a several-step signal transduction pathway leading to the nuclear localization and activity of the transcription factor Dorsal, which is highly homologous to mammalian NF-κB (see Refs. 17 and 18). The IL-1R1 cytoplasmic domain is homologous to that of the Drosophila receptor Toll, which provides the essential signal for embryo ventralization following fertilization (19–21). These homologous cytoplasmic regions of Toll and the IL-1R1 can be interchanged so that a chimera of the Toll cytoplasmic domain and the IL-1R1 extracellular domain is phosphorylated and degraded by IL-1 (22). The activity of Dorsal is controlled by the inhibitor protein Cactus, which like its homologous counterpart IκBα, is phosphorylated at discreet N-terminal sites, and is then destroyed by proteasomes, freeing Dorsal to migrate to the nucleus and begin transcription (22, 23). Key to the phosphorylation of Cactus in Drosophila is the unique Ser/Thr kinase Pelle, which is activated by its interaction with the membrane bound adapter protein Tube via death domains found on the N terminus of both proteins (24–26). While it appears that Tube recruits Pelle to the Toll receptor following activation, the molecular details of how this recruitment occurs and how Pelle is subsequently activated are currently unclear.

Cao et al. (5) have shown in IL-1R1-transfected HEK cells that IRAK is rapidly translocated to the IL-1R1 and becomes multiphosphorylated following IL-1 stimulation. In the present paper we show that a similar rapid translocation and phosphorylation of IRAK occurs following IL-1, but not TNFα, activation of normal human lung fibroblast MRC-5 cells. Following that activation IRAK largely disappears within minutes, and that destruction is prevented by proteasome inhibitors and by K-252b, a nonspecific kinase inhibitor.

EXPERIMENTAL PROCEDURES

Materials—MRC-5 cells were obtained from the American Type Culture Collection (ATCC CCL-171; Rockville, MD) and were grown in Williams medium with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). The LLL, IEAL, and LLNV proteasome inhibitors were obtained from Peptides International (Louisville, KY). The PP-2A phosphatase was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The IL-1α, TNFα, and soluble IL-1R1 were obtained from R & D Systems (Minneapolis, MN). R-252b was obtained from Alexis Biochemicals (San Diego, CA). The IκBα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to the IL-1R1 and IRAK were raised at Covance Research Products (Denver, PA) against the soluble IL-1R1 and against synthetic peptides of the indicated IRAK sequence (see Fig. 1A). The peptides were prepared at R & D Antibodies (Berkeley, CA) with an N-terminal Cys and coupled to bovine thyroglobulin as described previously (57). IL-1Rα was prepared at Merck and shown to prevent the high affinity binding of IL-1

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‡ To whom all correspondence should be addressed: Dept. of Inflammation Research, Merck Research Laboratories, P. O. Box 2000, R80N-A32, Rahway, NJ 07065. Tel.: 908-594-6838; Fax: 908-594-3111; E-mail: douglas_miller@merck.com.
§ The abbreviations used are: IL, interleukin; IL-1R1, Type I IL-1 receptor; IRAK, interleukin-1 receptor-associated kinase; PBS, phosphate-buffered saline; Cbz, benzoxycarbonyl.

2 A. Heguy, personal communication.
and IL-1β on MRC-5 cells and to prevent the subsequent production of PGE₂. All other reagents not specifically noted were obtained from Sigma.

Cell Growth and Sample Preparation—MRC-5 cells were subcultured weekly and for experiments they were grown to confluence in 150-cm² plates with medium supplemented with penicillin-streptomycin and Mycoticin (Life Technologies, Inc.). One 150-cm² plate was used for each immunoprecipitation. Prior to use, the medium was replaced with medium with penicillin-streptomycin, but no serum, for 24 h. Inhibitors dissolved in Me₂SO at 1000 × final concentration were added 40 min prior to cell stimulation. IL-1α or TNFα were added for 2–120 min at various concentrations, typically 10 ng/ml. For harvesting, the plates were immediately chilled in an ice tray, washed once with ice-cold PBS, and then scraped in ice-cold PBS containing 1 mM EDTA, 10 mM β-glycerophosphate, and 1 mM Na orthovanadate. The cells were centrifuged, and the cell pellet was lysed for 30 min on ice in 300 μl of a buffer (lysis buffer) containing 50 mM HEPES, pH 7.5, 0.5% Nonidet-P-40, 100 mM NaCl, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, and a protease inhibitor mixture containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin. The cells were spun for 20 min at 2500 rpm in a Beckman GPR centrifuge, and the supernatant was stored frozen at −80 °C.

Immunoprecipitation and Immunoblotting—The IRAK and IL-1R1 proteins were immunoprecipitated from the Nonidet-P-40-solubilized extract by the addition of 1–3 μl of antibody and incubation at 5 °C for 2–3 h on a rotator. Then 20 μl of a 50% slurry of prewashed protein A-agarose beads was added to each sample, followed by incubation for an additional 1 h at 5 °C. The samples were spun in a microcentrifuge and washed twice in lysis buffer without glycerol or protease inhibitors and then twice in a buffer (lysis buffer) consisting of 20 mM HEPES, pH 7.5, 20 mM MgCl₂, 20 mM β-glycerophosphate, and 1 mM sodium orthovanadate.

For immunoblotting, the beads were resuspended in 20 μl of 2× SDS sample buffer, separated on 15% SDS gels for 1 h, and transferred to nitrocellulose membranes with blocking, washing, and visualization by ECL techniques as described previously (58). Dilutions used in immunoblotting for the primary antibodies were 1:1000 for IRAK, 1:1000 for IL-1R1, and 1:3000 for the IL-1R1. For the secondary visualization step, horseradish peroxidase-protein A (Amersham Life Science, Inc.) was used at a 1:5000 dilution. Exposures were made from 5 s to 15 min. Films were scanned with a Quantity One densitometry system (PDI, Huntington Station, NY).

For multiple immunoblots on the same samples, the membranes were stripped immediately following the immunoblotting. To strip the membranes, they were washed four times with PBS containing 0.05% Tween 20 (PBS/Tween) and incubated for 30 min at 50 °C in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol. The stripped membranes were then washed 6 times with PBS/Tween and immobiloblotted for the next antibody. Each membrane was blotted for a maximum of three times.

RESULTS

IRAK Is Phosphorylated and then Disappears from MRC-5 Cells—IRAK is a multidomain protein containing an N-terminal death domain, a kinase domain, and an undetermined C-terminal domain, which is absent in Pelle (Fig. 1A). In this diagram the putative IRAK death domain homologous to that of Pelle is shown with the six-core α-helices (see Refs. 27 and 28), and the locations of the kinase subdomains are shown together with the locations of the 15 conserved kinase residues (5, 29). The locations of the introns are depicted by inverted triangles, based upon the sequence of the human gene within Xq28 as reported in GenBank™ (accession number U52112, deposited by M. Platter, D. Bauer, and B. Drescher). Those peptide regions used for the generation of polyclonal antipeptide IRAK antibodies in the present report are indicated with a bar. All three antisera immunoprecipitated a common protein band in unstimulated MRC-5 cells (Fig. 1B). When the MRC-5 cells were activated with IL-1α, this sharp IRAK band disappeared and a diffuse higher molecular weight band was formed as detected with the I700–712 antisera (Fig. 1B), analogous to the migration of the phosphorylated IRAK reported earlier (5) (see below). A similar pattern was seen when the I504/523 and 1364/388 antisera were used for immunoblotting, but the antibodies had substantially weaker immunoblotting activity (data not shown). No new immunodetectable IRAK protein fragments were detected in the IL-1α stimulated cells coincident with the disappearance of the IRAK protein band (data not shown).

3 S. M. Raju and D. K. Miller, unpublished results.

FIG. 1. Location and activity of antiIRAK antibodies. A, structure of IRAK showing the location of the death domain with its putative conserved α-helical subdomains, the kinase domain with its 11 subdomains and the 15 conserved amino acids, the location of the introns, and the peptide regions used for antibody generation. The peptide I364–388 comprises the activation loop found between subdomains VII and VIII (see text). B, immunoprecipitation of IRAK by the peptide antibodies shown in Fig. 1A and blotted with the I700–712 antisera. MRC-5 cells were stimulated for 0 or 5 min with 10 ng/ml IL-1α.
A detailed time course of IL-1α activation of the MRC-5 cells was performed, and the amount of IRAK associated with the IL-1R1 was observed by immunoprecipitation and immunoblotting with the corresponding antisera (Fig. 2A). In the absence of IL-1α stimulation, no IRAK was found associated with the IL-1R1. Within 30 s a small amount of IRAK was associated with the IL-1R1, and this small amount of IRAK migrated at about the same or at just a slightly slower rate as the unmodified IRAK observed in the IRAK intraperitoneal (Fig. 2A). This association of IRAK with the IL-1R1 was specific for IL-1 activation, because it was blocked by a 1000-fold higher concentration (10 μg/ml) of IL-1RA was mixed with the 10 ng/ml IL-1α prior to its addition to MRC-5 cells, the cells were incubated for up to 20 min at 37 °C, and the IRAK and its associated IL-1R1 were immunoprecipitated with the IL-1R1 antibody and blotted with a second IRK (Fig. 2B). This conversion of IRAK to a phosphorylated form was not observed in the MRC-5 cells in which IL-1RA blocked the IL-1 activation (Fig. 2B). All of the observed phosphorylations on IRAK were on Ser and Thr residues; the elution of all the phosphorylated IRAK protein could be shifted to the more rapid rate observed with the unphosphorylated IRAK following treatment with the Ser/Thr-specific phosphatase PP-2A (Fig. 2D; see Ref. 31).

Scans of the immunoblots showed that following a short lag the unphosphorylated IRAK disappeared with a t1⁄2 of about 2 min (bold line, Fig. 2E, top). This decrease probably reflected the conversion of unphosphorylated IRAK to the phosphorylated state, because it was reversed by phosphatase treatment whereas the faster migrating IRAK band found in the unstimulated cells was not labeled with [33P]PO4 (Fig. 2C). Multiple phosphorylations of the IRAK were observed in a time-dependent fashion with an increased phosphorylation observed at later times (Fig. 2A, 4- and 10-min time points versus 0.5 min) leading to a preponderance of the slowest migrating, most heavily phosphorylated forms at later times (see Fig. 2E; cf. Ref. 5). The percentage of the total IRAK immunoprecipitated that was phosphorylated was determined in the samples that had been immunoprecipitated by the IRAK or IL-1R1 antibodies (data from E, top panel).

The diffuse bands of IRAK observed immediately after IL-1 activation were due to phosphorylation of IRAK; in an in vitro [33P]PO4 kinase assay of IRAK immunoprecipitated from MRC-5 cells before and after IL-1 activation, the same immunoblottable diffuse IRAK bands could be labeled with [33P]PO4.
The amount of phosphorylated IRAK reached a maximum amount before 2 min and then began to rapidly decline (Fig. 2E, top panel). This pattern of the phosphorylated IRAK was observed regardless of whether the IRAK was detected by immunoprecipitation with an IRAK or an IL-1R1 antibody (Fig. 2E, top panel). If the amount of phosphorylated IRAK were normalized to that of the total IRAK detected to correct for the disappearance of IRAK, then maximal phosphorylation occurred later between 2 and 4 min (Fig. 2E, bottom). Hence, IRAK began to disappear as it was phosphorylated, so that the maximal amount of phosphorylated IRAK detected was a balance of the formation of phosphorylated IRAK and its degradation. The loss of IRAK observed within the Nonidet P-40-solubilized MRC-5 cells was not due to the translocation of IRAK to a detergent insoluble nuclear pellet, because immunochemical analysis of the detergent-insoluble pellets also showed the absence of IRAK (data not shown).

In contrast to the rapid disappearance of IRAK following IL-1 activation, the MRC-5 cell IL-1R1 is neither phosphorylated nor diminished in total amount (data not shown). If anything, the total amount of IL-1R1 that can be immunoprecipitated by IL-1R1 antibodies following IL-1 stimulation increases in amount during stimulation (see Fig. 2A, short exposure). The IL-1R1 seen by straight immunoblot or after immunoprecipitation of MRC-5 cell extracts by IL-1R1 consists of primarily two hazy bands. The same pattern is also found in the soluble IL-1R1 standard (missing the cytoplasmic tail and transmembrane region) that is expressed in mammalian cells (Fig. 2A). This heterogeneity most likely a result of variable glycosylation on the IL-1R1, which is known to have multiple glycosylation sites producing receptors with 23–35% total carbohydrate (32). The IL-1R1 communoprecipitated with IRAK comprises roughly 2% of the total IL-1R1 in the MRC-5 cells that can be immunoprecipitated by the IL-1R1. The form of the IL-1R1 associated with IRAK is principally that form that migrates the most slowly, perhaps with the largest amount of carbohydrate (Fig. 2A).

To compare the effects of IL-1 on IRAK activation and disappearance to that of that of the subsequently activated IκBα, aliquots of the IL-1α-activated samples (Fig. 2A) were immunoblotted with an antibody specific for IκBα. The results showed that the IκBα, like IRAK, was likewise phosphorylated by 1–2 min, and at the same time it largely disappeared from the cells by 10 min (Fig. 3, top panel). Comparison of the total amount of IκBα to the total amount of IRAK as detected by scans of immunoblots indicated that the t50 of disappearance of both proteins was about 4 min (Fig. 3, bottom panel). Hence, the disappearance from cells of both IRAK and IκBα occurred rapidly and in a parallel fashion following their phosphorylation.

**IRAK Phosphorylation Does Not Occur following TNFα Activation of MRC-5 Cells—**MRC-5 cells activated for 5 min with a dose response of IL-1 produced a graded response of IRAK activation, as measured by the formation of the phosphorylated band and the association with the IL-1R1, as well as a graded disappearance of IRAK (Fig. 4, top and middle panels). A comparable extent of IκBα phosphorylation and degradation in the same cells was observed (Fig. 4, bottom panel). In contrast, TNFα activation performed on a parallel series of plates showed that while the MRC-5 cells were activated as measured by the phosphorylation and degradation of IκBα (Fig. 4, bottom panel), there was no effect on either the phosphorylation or the degradation of IRAK (Fig. 4, top panel). Similarly in the TNFα-stimulated cells, there was no association of IRAK with the IL-1R1 (Fig. 4, middle panel). Hence, as noted earlier using IL-1R1 (Fig. 2B), the stimulus that induces IRAK association with the IL-1R1 and IRAK phosphorylation is specific for IL-1 stimulation and is not a result of an activated MRC-5 state.

**Proteasome Inhibitors Prevent IRAK Disappearance—**The addition to activated cells of various inhibitors of proteasome activity have enabled the accumulation of phosphorylated IκBα and have prevented its degradation. When effective concentrations of the peptide aldehyde inhibitors Cbz-Leu-Leu-Leu-H (LLL, 25 μM (33)), Cbz-Ile-Glu(04-butyl)-Ala-leucinal (IEAL, 80 μM (34)), or Cbz-Leu-Leu-norVal-H (LLNV, 50 μM (35)) were added to MRC-5 cells stimulated with IL-1α, IκBα was retained for up to 40 min (Fig. 5A, bottom panel). The destruction of IRAK within the same cells is similarly inhibited: proteasome inhibitors lead to a retention of the phosphorylated form of IRAK (Fig. 5A, top panel). The effects of the proteasome inhibitors did not prevent the disappearance of the majority of the nonphosphorylated IRAK (Fig. 5B, top panel), but rather prevented the loss of the phosphorylated IRAK (Fig. 5B, bottom panel).
Comparison of changes in the total amount of IRAK in the cells to that of IκBα showed that the proteasome inhibitors had a similar effect quantitatively on the retention of both proteins following IL-1α stimulation (Fig. 5C). Just as the proteasome inhibitors prevented IRAK destruction, they also increased the amount of IL-1R1 found associated with IRAK immunoprecipitates (Fig. 5D). The amount of the IRAK associated IL-1R1 doubled to a level of about 4% of the total immunoprecipitable receptor, but the type of IL-1R1 associating with IRAK (slower migrating form in SDS gels) remained the same. The total amount of IL-1R1 immunoprecipitated by the IL-1R1 antibody over the 40-min incubation period was not significantly changed, indicating that proteasome activity had no effects on IL-1R1 levels during IL-1 stimulation (data not shown).

**IRAK Degradation Is Inhibited by the Kinase Inhibitor K-252b**—The observation that it is the phosphorylated form of IRAK that is degraded by proteasomes (Fig. 5B) led to the prediction that an inhibitor of IRAK phosphorylation should prevent the degradation of IRAK. Because staurosporine analogs are known to be nonspecific inhibitors of a number of cellular kinases (36, 37), the effect of K-252b on IRAK phosphorylation and degradation was determined in IL-1α-stimulated MRC-5 cells. The formation of phosphorylated IRAK (as determined by the band shift on SDS gels) particularly in cells treated with 25 μM K-252b was delayed relative to the control, and at the same time the disappearance of IRAK was inhibited (Fig. 6, top panel). The association of IRAK with the IL-1R1 was, however, not inhibited by K-252b; in fact, the inhibited
cells showed a greater amount of IL-1R1 associated with IRAK following activation (Fig. 6, middle panel) similarly to what was observed when the degradation of IRAK was inhibited by proteasome inhibitors (Fig. 5). Despite the effects on IRAK, only modest effects were seen in the inhibition of IκBα degradation (Fig. 6, bottom panel). This suggests that K-252b differentially inhibits the kinase activity that results in IRAK hyperphosphorylation from that kinase activity producing IκBα phosphorylation.

Unstimulated IκBα, but Not IRAK, Is Regenerated during Prolonged IL-1 Stimulation—Previously it had been observed that prolonged IL-1 or TNFα incubation with sensitive cells first led to the degradation of IκBα, followed by its resynthesis and return within 60 min (8, 38). To compare the corresponding effects of continuous IL-1 administration on the presence of IRAK to that of IκBα, MRC-5 cells were incubated for up to 2 h with IL-1α. As is shown in Fig. 7 during prolonged exposure of IL-1, cellular IκBα was quickly lost, but returned to almost normal levels within 60 min (Fig. 7A, bottom panel; Fig. 7B, middle panel). If the proteasome inhibitor LLL was present, the initial loss of IκBα was delayed, and a considerable amount of the IκBα was retained as the phosphorylated IκBα (Fig. 7A, bottom panel). The resynthesized IκBα, however, was nonphosphorylated as seen by the absence of the phosphorylated IκBα band with LLL at 60 and 120 min (Fig. 7A, bottom panel). That the newly synthesized IκBα was unstimulated was seen by the subsequent addition of TNFα to dishes of MRC-5 cells that had been continuously stimulated for 105 min with IL-1α. After 15 min of TNFα exposure the newly synthesized IκBα disappeared unless LLL was present in the incubation, at which point much of the IκBα was present as the phosphorylated form (Fig. 7A, bottom right set of panels). In contrast, the readaptation of more IL-1α had no effect on the phosphorylation or disappearance of IκBα, indicating that the cells were down-regulated with respect to IL-1-mediated activity.

The long term effects of the IL-1 treatment on IRAK in the MRC-5 cells were much less dramatic than those of IκBα. While most of the IRAK was phosphorylated and removed by 12–30 min, a small amount of both phosphorylated and unphosphorylated IRAK persisted for as long as 120 min (Fig. 7A, left and middle panel). Addition of LLL in the incubation increased the amount of phosphorylated IRAK, but had no effect on the unphosphorylated IRAK (Fig. 7A, top panel). It thus appeared that there was a continuous production of a newly synthesized pool of IRAK that became phosphorylated and subsequently degraded unless inhibited by the presence of LLL. IL-1R1 levels associated with IRAK gradually declined after 12–60 min unless LLL was present (Fig. 7A, middle panel; Fig. 7B, bottom panel; compare with Fig. 7B, top panel). As expected from the prior presence of IL-1 with these cells and the lack of effects of TNFα on IRAK, analysis of the IRAK in the cells that had received additional TNFα or IL-1α at 105 min showed no difference from the cells that had continuous IL-1α exposure (Fig. 7A, top right set of panels).

DISCUSSION

In the present report we confirm in a normal human fibroblast line the previous observations in IL-1R1-transfected HEK cells that IRAK associates with the IL-1R1 and becomes phosphorylated after IL-1 stimulation (5). We now find that following its phosphorylation IRAK becomes a target for cellular proteolysis by proteasomes in a similar manner to that seen previously with the phosphorylation and subsequent proteasome destruction of IκBα. IRAK phosphorylation and its destruction is specific for activation of the IL-1R1: prevention of IL-1R1 activation by the specific IL-1 receptor antagonist IL-1RA or independent cellular and IκBα activation by other stimulators such as TNFα have no effect on the phosphorylation and destruction of IRAK. Inhibition of IRAK phosphorylation, like inhibition of proteasome activity, does not, however, prevent association of IRAK with the IL-1R1, indicating that translocation of IRAK to the IL-1R1 occurs prior to or inde-
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A, in the absence of stimulation, IRAK is unphosphorylated and separate from the IL-1R1, which is depicted on cells in a lower and a higher glycosylation state. B, following IL-1 stimulation the majority of the IRAK translocates to a fraction of the IL-1R1 with a higher glycosylation state where it becomes phosphorylated. The now activated IRAK can activate a downstream ubiquitin-dependent kinase which phosphorylates IκBα. The phosphorylation of both IRAK and the IκBα results in their degradation by proteosomes. NF-κB once freed of its inhibitor IκBα, translocates to the nucleus where it can begin transcription. TNFα through its receptor can also activate the kinase-phosphorylating IκBα independently of IL-1, in which case there is no activation of IRAK, no association with the IL-1R1, no phosphorylation, and no degradation of IRAK. C, following continuous IL-1 exposure for up to 2 h, the majority of IRAK has disappeared, but a small amount of activated IRAK remains associated with the IL-1R1 as seen by its coimmunoprecipitation. A small amount of unphosphorylated IRAK remains unassociated with the IL-1R1. IκBα is resynthesized by ~60 min and is insensitive to further IL-1 activation, but it is sensitive to TNFα-induced phosphorylation and degradation.

The low phosphorylation state of IRAK observed when it initially translocates to the IL-1R1 (see Fig. 2A and Ref. 5) suggests that the phosphorylation of IRAK occurs subsequent to its translocation. Either it is the association of IRAK with the IL-1R1 perhaps together with the IL-1 accessory protein (see Ref. 5) that activates IRAK producing its autophosphorylation, or there is present another kinase that cotranslocates to the same IL-1R1-IRAK complexes that can phosphorylate IRAK in a K-252b-inhibitable fashion.

The model for IRAK behavior following IL-1 activation is diagrammed in Fig. 8. In the unstimulated cell state (Fig. 8A) IRAK is a presumably cytoplasmic inactive kinase (4) that is not associated with the IL-1R1. The IL-1R1 itself exists on the membrane as a mixture of receptors of differing glycosylation state (32). In Fig. 8A the IL-1R1 is depicted as containing either five or two glycosylation chains, although the exact molecular differences between these forms are unknown. When IL-1 occupies the IL-1R1, IRAK translocates to the most highly glycosylated form of the IL-1R1 (Fig. 8B), where it is also found associated with the IL-1 accessory protein (data not shown). As IRAK becomes associated with the IL-1R1, it becomes increasingly phosphorylated and simultaneously activated (4). Because roughly three fuzzy bands can be seen following activation (Fig. 2A and Ref. 5), IRAK is depicted with three phosphates attached, although exactly how many residues are phosphorylated, and to what extent, is not known. Based upon the conservation of sequence of IRAK with the Drosophila Pelle kinase (5) and its known requirement for its N-terminal death domain for activation (25, 26), we assume that it is this domain of IRAK that is responsible for its association with the cytoplasmic domain of the IL-1R1.

Both IL-1 and TNFα stimulate a ubiquitin-dependent kinase that is responsible for the phosphorylation of IκBα (7). Presumably this IκBα-kinase is activated directly or indirectly through unknown intermediary kinases by IRAK as well as by kinases associated with the TNFs Type I (10, 11) and/or Type II receptors (9, 40). The resultant IκBα phosphorylation tags IκBα for its subsequent destruction by proteasomes (8, 13−15, 41) and enables the subsequent activation and nuclear translocation of NF-kB (see Ref. 42). The phosphorylated IRAK is also largely destroyed by proteasome activity at a rate comparable with that of IκBα. Degradation of both IRAK and IκBα is retarded by proteasome inhibitors, but proteasome inhibitors do not prevent the association of IRAK with the IL-1R1 nor the subsequent conversion of the nonphosphorylated IRAK to the phosphorylated form. It is not clear at this point how closely correlated the activation of IRAK is relative to its total phosphorylation. The addition of 25 μM K-252b inhibits IRAK phosphorylation to the extent that IRAK is largely degraded by 10 min, yet it has little effect on the subsequent activation of the IκBα kinase as measured by the phosphorylation and degradation of IκBα (Fig. 6). This suggests that IRAK may still be activated (perhaps even phosphorylated on its activation loop, see Ref. 43) to a sufficient extent to lead to subsequent IκBα phosphorylation, but not so extensively phosphorylated that it is recognized for proteasome destruction. Alternatively, the signaling pathway may be so amplified that a small amount of IRAK activity still formed at 25 μM K-252b is sufficient to activate enough of the downstream kinases to phosphorylate IκBα. Unfortunately the amount of IRAK activity in the MRC-5 cells using artificial exogenous substrates is too low to accurately correlate its activity with the extent of its phosphorylation (data not shown).

Clearly there are at least two forms of IRAK present within the cells. The first and most prominent form of IRAK is that form that associates with the IL-1R1, becomes phosphorylated, and is then degraded. Despite the destruction of the majority of IRAK following IL-1 stimulation, a significant amount of IRAK remains undegraded, replaced perhaps by new synthesis, to enable the prolonged immunoprecipitation of the IL-1R1. But the steady state amount of IRAK during prolonged IL-1 stimulation is probably only 20% of the original amount (see Figs. 5C and 7B, top panels), yet the amount of the IL-1R1 remaining associated with IRAK is almost unchanging (Figs. 5D and 7B, bottom panels). This suggests perhaps that the IL-1R1 comes together in an unknown complex containing a number of IL-1R1 molecules associated with IRAK. Perhaps once formed, this IL-1R1 complex is stable so that only a small amount of IRAK need be present to enable the immunoprecipitation of the whole complex. Proteasome inhibitors do not change this stoichiometry; proteasome inhibitors lead to the retention of double the amount of IRAK as well as double the amount of IL-1R1 that can be immunoprecipitated (see Fig. 7B, top and bottom panels).

The number of IL-R1 molecules that we observe to be immunoprecipitated in these experiments is comparable with earlier reports of IL-1R1 molecules that need to occupied for cell activation. MRC-5 cells contain about 3000 high affinity IL-1 receptors/cell (KD ~10 pm (44)), a level which is among the highest of any cell lines (see Ref. 45). Occupation by IL-1 of fewer than 20 receptors/cell is sufficient for activation (46), resulting in only 2−3% of the available receptors that need to be utilized (see Ref. 47). In line with these former observations, we have observed that from a plate containing 5 × 10⁵ MRC-5 cells we immunoprecipitate with anti IL-1R1 antibodies an amount of IL-1R1 comparable with 1 ng of sIL-1R1 (estimated molecular weight: 60,000, (48); see, for example, Fig. 2A). This corresponds to an immunoprecipitation of 2500 IL-1R1 molecules/MRC-5 cell. Following IL-1 stimulation and immunoprecipita-
tion by IRAK antibodies, only 2–4% of these receptors become associated with IRAK. Because apparently only a portion of the most highly glycosylated form of the IL-1R1 becomes associated with IRAK, those IL-1R1 molecules associating with IRAK are a distinct subset of the total IL-1R1 molecules in the MRC-5 cells. It is possible that different glycosylation or other unidentified posttranslational modifications yield IL-1R1 molecules of varying affinity for IL-1 on the extracellular surface or for IRAK on the cytoplasmic surface.

A second form of IRAK comprises that small percentage of IRAK that remains as unphosphorylated, unstimulated IRAK (see e.g. Figs. 5A and 7A, top panels). It is not found associated with IL-1R1 immunoprecipitates (Fig. 2A and data not shown), suggesting that it is not associated with the IL-1R1 at any time during the IL-1 stimulation. The amounts of this form of IRAK do not change with time, nor are they increased in the presence of proteasome inhibitors. It is possible that this unmodified IRAK represents a pool of unphosphorylated IRAK sequestered in a different part of the cell. Alternatively this IRAK form may be structurally different, because it has varying posttranslational modifications or is translated from an alternatively transcribed mRNA.

While TNFα clearly activates MRC-5 cells to a similar extent as IL-1, it is unknown which is the responsible TNF receptor in the MRC-5 cells. Most likely it is the 55-kDa TNFR1 that is active, since it is known that the TNFR1 is more widespread among cell types and that the TNFR1 is thought to be associated with the activation of nonlymphoid cells (see Refs. 49 and 50 for reviews). Furthermore, when cells containing both types of TNF receptors were activated with TNF mutants specific for individual TNF receptors (51), only the TNFR1 produced an activation of NF-κB (52). This kinase has recently been identified as the receptor interacting protein, which also interacts with IRAK, those IL-1R1 molecules associating with IRAK are not phosphorylated, and those IL-1R1 molecules associating with IRAK may act as a substrate for IRAK activity. IRAK on the cytoplasmic surface.

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