IL-1-induced Post-transcriptional Mechanisms Target Overlapping Translational Silencing and Destabilizing Elements in \( \kappa B \) mRNA*\(^5\)

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The inflammatory cytokine IL-1 induces profound changes in gene expression. This is contributed in part by activating translation of a distinct set of mRNAs, including \( \kappa B \), as indicated by genome-wide analysis of changes in ribosomal occupancy in IL-1 \( \alpha \)-treated HeLa cells. Polysome profiling of \( \kappa B \) mRNA and reporter mRNAs carrying its 3′ UTR indicated poor translation in unstimulated cells. 3′ UTR-mediated translational silencing was confirmed by suppression of luciferase activity. Translational silencing was unaffected by replacing the poly(A) tail with a histone stem-loop, but lost under conditions of cap-independent internal initiation. IL-1 treatment of the cells caused profound shifts of endogenous and reporter mRNAs to polysome fractions and relieved suppression of luciferase activity. IL-1 also inhibited rapid mRNA degradation. Both translational activation and mRNA stabilization involved IRAK1 and -2 but occurred independently of the p38 MAPK pathway, which is known to target certain other post-transcriptional mechanisms. The translational silencing RNA element contains the destabilizing element but requires additional 5′ sequences and is impaired by mutations that leave destabilization unaffected. These differences in function are associated with differential changes in protein binding in vitro. Thus, rapid degradation occurs independently of the translational silencing effect. The results provide evidence for a novel mode of post-transcriptional control by IL-1, which impinges on the time course and pattern of IL-1-induced gene expression.

Members of the IL-1 family of cytokines are intricately involved in inflammatory and immune responses (1). The founding members IL-1 \( \alpha \) and -\( \beta \) exert their multiple activities by binding to the same species of heterodimeric cell surface receptors present on virtually all nucleated cells. Consequences of their activation include changes in expression of numerous genes through activation of NF-\( \kappa B \) and the JNK and p38 MAPK cascades (2–5). In addition to transcriptional activation, post-transcriptional mechanisms have been reported to contribute to these changes. mRNA stabilization in response to IL-1 as well as LPS and other inflammatory activators depends on the p38 MAPK/MK2 pathway, which affects proteins that control degradation of mRNAs through selective binding to AU-rich elements (AREs)\(^3\) (6, 7). The PI3K and JNK pathways have been reported as well to lead to stabilization of mRNAs (8, 9). In addition, IL-1 induces stabilization of several mRNAs independently of TRAF6/p38/MK2 through an as yet unidentified signaling pathway downstream of IRAK1 (10).

The amount of a protein synthesized by the cell in a given condition is finally controlled at the level of translation by mechanisms related to those controlling mRNA degradation (11). Translational control of gene expression can affect most RNAs as an adaptation of general protein synthesis to cell growth stimulation or stress conditions, or it can specifically target a small group of mRNAs (12, 13). This study provides an example for the latter. Taking a microarray-based approach to investigate the effect of IL-1 on translation, we obtained evidence that a set of functionally related mRNAs is translationally activated by IL-1. In one of them, the mRNA of \( \kappa B \), a specific IL-1-controlled translational silencing element was identified. \( \kappa B \) (NF\( \kappa B \)IZ, also termed MAIL or INAP) was identified as a protein induced by LPS or IL-1 (14–16) but not induced (14, 16) or only weakly induced by TNF (17). \( \kappa B \) is localized in the nucleus and, unlike classical \( \kappa B \) proteins, is not degraded upon cell stimulation (17). Although negative effects on NF-\( \kappa B \) activity have been observed (16, 17), \( \kappa B \) was reported to potentiate LPS-induced IL-6 formation (15). Correspondingly, deletion of the \( \kappa B \) gene in mice resulted in impaired expression of IL-6 as well as GM-CSF and the p40 subunit of IL-12 (18). \( \kappa B \) mice also develop a severe atopic dermatitis-like disease (19) and show defective development of the IL-17-producing TH17 cell subset (20). Further studies confirmed that \( \kappa B \) can suppress certain genes while being required for the expression of others (21–23). The comparatively poor induction of \( \kappa B \) by TNF might explain in part differences in the induction of genes between TNF and IL-1 or LPS, as has been observed for IL-1-specific induction of neutrophil gelatinase-associated lipocalin and \( \beta \)-defensin 2 in a lung epithelial cell line (24, 25). Our

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results suggest that a translational control mechanism activated by IL-1 facilitates expression of IκBζ, which in turn controls expression of a subset of IL-1-regulated genes.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Expression plasmids for the β-globin construct used in reporter assays (petestBBB) (26), constitutively active MKK6, MEKK1, and dominant negative p38 MAPK have been described earlier (27). Plasmids for expressing wild-type IRAK1 and IRAK2 were obtained from Mark Windheim (Medical School Hannover) with kind permission of Philip Cohen (University of Dundee). To interfere with IL-1 signaling, the death domain of IRAK1 was expressed from plasmid pFl-IRAK1-DD (28, 29), a gift of Detlef Neumann (Medical School Hannover) and Michael Martin (Justus-Liebig-University Giessen). The 3′ untranslated region (UTR) of human IκBζ (nucleotides nt 2273–3885, numbering according to NM_031419) and fragments thereof as well as a fragment of the murine 3′ UTR (nt 2156–2361, NM_001159395) were amplified by RT-PCR from total RNA of HeLa cells or RAW264.7 cells, respectively. Primer sequences are available on request. Fragments were inserted into the BglII site 3′ of the β-globin coding region in ptestBBB or its derivative, ptestBBB-hsl, in which the polyadenylation signal was destroyed and the stem-loop and 3′ sequences of the human histone 1.3 gene were inserted to express non-polyadenylated mRNA (30, 31). The IκBζ 3′ UTR was also cloned into the Spel and HindIII sites 3′ of the firefly luciferase coding region in pMir-Report (Ambion). Point mutations were introduced by using the QuikChange kit (Stratagene). Fragments of the IκBζ 3′ UTR were cloned into pBluescript to obtain templates for in vitro transcription of RNAs applied in electrophoretic mobility shift assays (for details, see Ref. 31). Renilla luciferase was expressed using the plasmid phRL-TK (Promega). The bicistronic vector pB5RlucPluc (32) was a gift of Hansjoerg Hauser (HZI Braunschweig). RT-PCR-generated fragment nt 2273–2479 of IκBζ was cloned into the NotI site 3′ of the firefly luciferase ORF or into the SacI/Sall sites 3′ of the Renilla luciferase ORF, replacing the polio virus internal ribosome entry site (IRES).

**Cells and Materials**—HeLa cells constitutively expressing the tetracycline-controlled transactivator protein (33) were cultured and transfected by the calcium phosphate method as detailed earlier (31). Cells were stimulated with recombinant human IL-1α (Promocell). Degradation kinetics of plasmid-expressed chimeric β-globin mRNA was determined using the tet-off system. Transcription was stopped by doxycycline, and total RNA was isolated at different time points thereafter, followed by reverse transcription and quantitative PCR (RT-qPCR) determination of mRNA amounts (see below).

**SDS-PAGE and Western Blot**—Total cell lysate was separated by SDS-PAGE (12.5% acrylamide), and proteins were blotted onto nitrocellulose membranes. Equal loading and protein transfer were controlled by Ponceau S staining. After blocking with 5% dried milk in Tris-buffered saline, the blots were hybridized to antibodies against the FLAG epitope (Stratagene) or p38 MAPK (BD Biosciences) and peroxidase-coupled secondary antibodies. Chemiluminescence was detected by the LAS 3000 imaging system (Fujifilm).

In **Vitro Transcription and Electrophoretic Mobility Shift Assays**—The 32P-labeled RNA fragments and unlabeled RNA fragments used as competitors were synthesized with T7 RNA polymerase from linearized pBluescript plasmids. Purification of RNA probes, incubation with cytoplasmic extracts, UV cross-linking, and separation of the RNA-protein complexes was carried out as described previously (31).

**Sucrose Gradient Fractionation and RNA Isolation**—To prepare polysomes, 1.25 × 10^7 HeLa cells were rinsed and scraped in ice-cold PBS containing cycloheximide (0.1 mg/ml). Subsequent steps were carried out in the cold. After pelleting by centrifugation at 500 × g for 7 min, the cells were resuspended in extraction buffer (20 mM Tris-HCl, pH 8.0, 140 mM KCl, 0.5 mM DTT, 5 mM MgCl2, 0.5% Nonidet-P40, 0.1 mg/ml cycloheximide, and 0.5 mg/ml heparin) and incubated for 5 min on ice. Extracts were centrifuged for 10 min at 12,000 × g. Approximately 0.5 ml of supernatant was layered onto a 12-ml linear sucrose gradient (10–50% sucrose (w/v) in 20 mM Tris-HCl, pH 8.0, 140 mM KCl, 0.5 mM DTT, 5 mM MgCl2, 0.1 mg/ml cycloheximide, and 0.5 mg/ml heparin) and centrifuged at 4 °C in an SW40Ti rotor (Beckman, Palo Alto, CA) at 35,000 rpm without brake for 80 min (120 min in experiments examining the distribution of β-globin reporter mRNAs). The gradients were collected into 12 1-ml fractions, and absorbance profiles at 260 nm were recorded (ISCO, UA-6 detector). 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol alcohol were added to the probes for overnight precipitation at −20 °C. RNA was purified using Nucleospin RNAII tubes (Macherey & Nagel) following the manufacturer’s protocol. After elution, the RNA was precipitated by adding LiCl to a final concentration of 2 m and resolubilized in 15 μl of RNase-free water. RNA concentration was determined, and the samples were stored at −80 °C.

**mRNA Detection**—Northern blot analysis of β-globin and GAPDH mRNA was performed as previously described (31, 34), using digoxigenin-labeled antisense probes. Reverse transcription and quantitative PCR were carried out essentially as described earlier (35). TaqMan probes (Applied Biosystems, assay-IDs as follows: NFKBIA, Hs00153283_m1; IL-8, Hs00174103_m1) were used with TaqMan® Fast Universal PCR Master Mix (2×) (Applied Biosystems). A custom-made TaqMan gene expression assay was used for quantification of rabbit β-globin mRNA. SYBR Green-based detection was applied to quantify mRNAs using the following primers: IκBζ, 5′-gcacaactcatcaaggc (sense) and 5′-tgggcttctgtagtaggg (antisense); ZC3H12A, 5′-gctacggagacagtt (sense) and 5′-tacctacctacaaggg (antisense); GAPDH, 5′-tcaaatggtagaagag (sense) and 5′-atgtgagagatagc (antisense). Values with S.D. of >0.2 cycles were excluded from the analysis. For calculation of luciferase protein/mRNA ratios, luciferase activity (in relative light units) was divided by RNA units, calculated as 2^{−ΔΔCT} × 10^5, based on GAPDH mRNA determined as housekeeping mRNA.

**Luciferase Reporter Assays**—HeLa cells were transfected with luciferase expression vectors (see above). For determining the effect of IL-1, cells originating from the same transfected cul-
ture dish were reseeded into parallel cultures, incubated overnight, and then stimulated with IL-1α or left untreated. The cells were lysed, and firefly luciferase activity was determined as described (36). *Renilla* luciferase activity was determined with a Lumat LB9501 luminometer (Berthold) by mixing cleared lysate with potassium phosphate buffer (100 mM, pH 7.8) containing 500 mM NaCl, 1 mM EDTA, and 4 μM coelenterazine (Promega).

**Microarray Analysis**—Cy3-labeled cRNA was prepared from RNA of pooled sucrose gradient fractions, subjected to quality control, and analyzed using whole human genome oligonucleotide microarrays (G4112F, ID 014850, Agilent Technologies) in essence as described (37). Data were filtered according to a stringent multistep approach that accounted for 1) quality of the measurements (hybridization performance), 2) consistency among replicate assays, 3) intensity range, and 4) -fold change values (see also the legend to Table 1). Data from poorly annotated/characterized transcripts were excluded from analysis. A detailed protocol is available upon request.

**RESULTS**

**IL-1 Induces a Selective Increase in Ribosome Occupancy of Certain mRNAs**—To find out if IL-1 affects gene expression at the level of translation, an initial screen was performed in HeLa cells for IL-1-induced changes in ribosomal occupancy of mRNAs. Cytoplasmic lysates of unstimulated and IL-1-stimulated cells were subjected to polysome fractionation on sucrose gradients (Fig. 1A). IL-1 treatment did not exert a general effect on the distribution of ribosomes and their subunits (not shown). RNA isolated from fractions 2–4 (containing free ribosomes/subunits and untranslated mRNAs) and from fractions 7–10 (containing polysomes) was pooled. The relative amounts of individual mRNAs in the two pools were determined using

**Table 1:** mRNAs showing increased ribosome occupancy in response to IL-1α

| Gene symbol | Full gene name                                                                 | Increase -fold |
|-------------|-------------------------------------------------------------------------------|----------------|
| NFKBIZ      | Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ζ   | 20.0           |
| NFKBID      | Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, δ   | 7.6            |
| CXCL3       | Chemokine (CXC motif) ligand 3                                               | 6.4            |
| ZC3H12A     | Zinc finger CCCH-type containing 12A                                        | 4.8            |
| CXCL2       | Chemokine (CXC motif) ligand 2                                              | 4.2            |
| IL-6        | Interleukin 6 (interferon, β2)                                              | 3.7            |
| RC3H1       | Ring finger and CCCH-type zinc finger domains 1                             | 2.9            |
| CLK1        | CDC-like kinase 1                                                           | 2.6            |
| PHLDB2      | Pleckstrin homology-like domain, family B, member 2                         | 2.6            |

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an oligonucleotide microarray that covers about 31,000 human genes. In two independent assays, a small group of mRNAs consistently showed a more than 2-fold increase in the ratio of signals obtained for the pooled fractions 7–10 (translated) over pooled fractions 2–4 (untranslated). Table 1 lists those nine mRNAs (of 9800 mRNAs of annotated genes amenable to analysis) in which the mean increase was more than 2.5-fold.

In subsequent experiments, the distribution of four of the mRNAs listed in Table 1 was analyzed by RT-qPCR for individual fractions. A pronounced shift from untranslated to translated fractions in response to IL-1 was confirmed for the mRNA of IκBα (Fig. 1B). Marked shifts were also observed for mRNAs of NFKBID (also named IκBβ or IκBNS), IL-6, and ZC3H12A (also named MCPIP1) (supplemental Fig. S1). GAPDH mRNA was analyzed as a housekeeping mRNA control (Fig. 1B). The distribution of this mRNA did not shift to the translated fractions but even slightly to untranslated fractions. Most of the mRNAs that shift to translated fractions are also induced by IL-1. In a control experiment, it was observed that the mere increase in amount of a given mRNA per se does not result in a change in the relative distribution during density gradient centrifugation (not shown). Altered distribution might have been a general feature of IL-1-induced mRNAs. This was ruled out by analyzing the mRNA of IκBα, which is also rapidly induced by IL-1. Unlike IκBζ mRNA, the distribution of IκBα mRNA was not affected by IL-1 (Fig. 1B). These data indicated selectively
enhanced translation of a distinct set of mRNAs, including that of IκBζ in IL-1-stimulated cells.

**The IκBζ 3′ UTR Harbors an IL-1-controlled Translational Silencing Element**—The mRNA region essential for low ribosome occupancy of IκBζ mRNA could be localized to the 3′ UTR by using a β-globin mRNA-based reporter system (scheme in Fig. 2A). Northern blot analysis showed that β-globin mRNA without insertion is mainly localized in polysome fractions (Fig. 2A). Its distribution is not affected in response to IL-1 (not shown). Introducing the IκBζ 3′ UTR downstream of the β-globin stop codon resulted in a shift in the distribution of the chimeric mRNA to untranslated fractions (Fig. 2A), thus resembling the distribution of endogenous IκBζ mRNA from unstimulated cells. IL-1 treatment of the cells caused a shift back to polysome fractions, again corresponding to the effect of IL-1 on endogenous IκBζ mRNA. Preferential localization of β-globin-IκBζ chimeric mRNA in untranslated fractions under basal conditions and redistribution to polysome fractions in response to IL-1 was confirmed by RT-qPCR quantification of samples from an independent assay (Fig. 2B). Thus, the 3′ UTR of IκBζ imposes low ribosome occupancy and its reversal by IL-1 on a heterologous reporter mRNA, suggesting that the IκBζ 3′ UTR harbors a strong translational silencing element that is controlled by IL-1.

To find out if indeed altered distribution in the polysome gradients reflects a change in translation, the IκBζ 3′ UTR was inserted 3′ of the firefly luciferase coding region in a plasmid expressing luciferase under control of the CMV promoter (Fig. 2C, scheme). Luciferase activity and mRNA were determined in parallel in HeLa cells transfected with the vector lacking or containing the IκBζ 3′ UTR. The data in Table 2 indicate that the insertion caused a striking decrease of the ratio of luciferase activity over RNA. Furthermore, IL-1 treatment induced an increase in the activity/RNA ratio for the transcript containing the IκBζ 3′ UTR, thus partially reversing its repressing effect (Fig. 2C). This result confirms that the IκBζ 3′ UTR contains a translational silencing element whose function is compromised in response to IL-1.

**Translational Activation Is Not a General Consequence of IL-1-induced mRNA Stabilization**—Ectopically expressed murine IκBζ mRNA has been reported to be rapidly degraded in unstimulated cells and stabilized in response to IL-1 (38). Insertion of the human IκBζ 3′ UTR into the β-globin mRNA, which by itself has a long half-life and is widely used for identification of destabilizing RNA elements (26, 27, 39), results in rapid degradation of the chimeric mRNA in HeLa cells (Fig. 3A). IL-1 treatment induced a marked stabilization, as observed for other short lived mRNAs (e.g. see Refs. 27, 30, and 41). Therefore, we hypothesized that the observed shift in polysome gradients was mechanistically related to or a general consequence of mRNA stabilization. This was ruled out, however, by the fact that no shift in the polysome gradient fractions was detected for an established target of IL-1-induced stabilization, the mRNA of IL-8 (27, 31) (Fig. 3B; note the preferential location in untranslated fractions) or for a reporter mRNA that contained the ARE of IL-8 mRNA, which mediates control of stability (not shown). Furthermore, no IL-1-induced increase in luciferase activity was observed with a reporter construct containing the IL-8 ARE instead of the IκBζ 3′ UTR (supplemental Fig. S2).

**IL-1 Affects Translation of IκBζ 3′ UTR-containing mRNAs**

Independently of the p38 MAPK Pathway—IL-1 can stabilize mRNAs through activation of the p38 MAPK/MK2 signaling cascade (27) or independently thereof (41). Expressing a dominant negative mutant of p38 MAPK (p38ΔAGF), which is effec-

### TABLE 2

| Suppression of luciferase expression by the IκBζ 3′-UTR |
|--------------------------------------------------------|
| Shown in column 2 is the ratio of luciferase activity/RNA. Luciferase activity and mRNA were quantified as detailed under "Experimental Procedures" for HeLa cells transfected with an expression vector for firefly luciferase without (Luc) or with the IκBζ 3′ UTR (Luc-IκBζ). Expt., experiment. |
| Luc | Luc-IκBζ | Suppression |
|-----|----------|-------------|
| Expt. 1 | 27.5 | 0.96 | 28.7 |
| Expt. 2 | 38.0 | 0.94 | 40.3 |
| Expt. 3 | 57.7 | 1.31 | 44.0 |
| Expt. 4 | 31.8 | 0.93 | 34.3 |
| Mean | 36.8 | | |
| S.D. | 6.7 | | |

**FIGURE 3.** The IκBζ 3′ UTR and the IL-8 ARE differ in mediating IL-1-induced post-transcriptional control. A, HeLa cells expressing the β-globin-IκBζ 3′ UTR chimeric mRNA (see scheme in Fig. 2A) were incubated without or with IL-1 for 30 min, followed by the addition of doxycycline (3 μg/ml) to stop transcription from the tet-off promoter. RNA isolated at that time (0 min) and 30 or 60 min later was analyzed by RT-qPCR for the amount of chimeric mRNA. Results are expressed in percentage of the amount at 0 min (mean ± S.D. error bars of three independent experiments). B, polysome profiles of endogenous IL-8 mRNA were generated for cells incubated without or with IL-1 for 1 h. One of two independent experiments with similar results is shown.
tive in reversing the IL-1-induced stabilization of mRNAs (27), did not interfere with the IL-1-induced shift of the chimeric mRNA to polysome fractions (Fig. 4A). Furthermore, expressing a constitutively active mutant of the p38 MAPK-selective upstream kinase, MKK6 (MKK62E), which stabilizes IL-8 mRNA (27, 31), did not shift the distribution of the β-globin-IkBζ chimeric mRNA to polysome fractions but even had a slight reverse effect (supplemental Fig. S3A). MEKK1Δ, a truncated form of the MAPK kinase MEKK1, which activates the p38, JNK, ERK, and NF-κB pathways (see Ref. 36 and references therein), also failed to recruit the reporter mRNA to poly-

somes (supplemental Fig. S3B). This confirms the lack of involvement of p38 MAPK in the translational effect of IL-1 and argues against a role of the other pathways activated by MEKK1 alone or in combination. Active MKK6 did not result in stabilization of the chimeric mRNA, and expression of dominant negative p38 MAPK did not interfere with stabilization induced by IL-1 (supplemental Fig. S3C). Overexpressing the upstream components of IL-1 signaling, IRAK1 and -2, could mimic both the translational (Fig. 4, B and C) and the stabilizing effect of IL-1 (supplemental Fig. S4A), whereas expressing a truncated form of IRAK1 which acts in a dominant negative manner (28, 29), interfered with both effects of IL-1 (supplemental Fig. S4, B and C). These data suggest that signaling pathway(s) inducing these effects diverge from the previously described p38 MAPK-dependent pathway at a step downstream of IRAK.

The Translational Silencing Element (TSE) in IκBζ mRNA Overlaps but Is Not Identical with an IL-1-controlled Destabilizing Element (DSE)—The relationship between translational silencing and destabilization mediated by the IκBζ 3′ UTR was explored further by identifying the RNA regions essential for these effects. Analysis of fragments of the IκBζ 3′ UTR using the β-globin reporter system revealed that nt 2273–2425 are sufficient for both rapid mRNA degradation and translational silencing (Fig. 5, A and B). Further truncation of the 3′ end significantly impaired destabilization and abolished translational silencing (fragment 2273–2403 in Fig. 5, A and B). Further truncation of the 5′-end (nt 2311–2479) also abolished the translational silencing effect, indicating that the fragment containing nt 2273–2425 is the smallest fragment harboring the complete TSE. In contrast, chimeric mRNAs were still rapidly degraded, even when containing only nt 2333–2425 (Fig. 5, A and B). Only further shortening to nt 2378 abolished destabilization. Thus, although sequences between nt 2273 and 2333 are essential for translational silencing, they are dispensable for the activity of the DSE.

**FIGURE 4.** Evidence against involvement of p38 MAPK in IκBζ 3′ UTR-dependent translational activation and stabilization induced by IL-1. A, polysome profiles were obtained for β-globin-IkBζ 3′ UTR mRNA in cells co-transfected with empty vector (con) or with an expression vector for FLAG-tagged dominant negative p38 MAPK (dn-p38) and left untreated or incubated with IL-1 for 1 h as indicated. Expression of dominant negative and total p38 MAPK was determined by Western blot. Results were confirmed in two independent experiments. B, polysome profiles for β-globin-IkBζ 3′ UTR mRNA in cells co-transfected with empty vector or equal amounts of plasmids expressing IRAK1 and -2 (IRAK1 +2). C, cells were transfected with a luciferase-IκBζ 3′ UTR construct (see Fig. 2C) and either stimulated with IL-1 for 4 h or co-transfected with expression vectors for IRAK1 or for IRAK2. Luciferase activity was normalized to co-expressed Renilla luciferase. Results are expressed as -fold increase over the activity in untreated cells co-transfected with empty vector (mean ± S.D. (error bars) of two independent experiments).
In agreement with the functional importance of the TSE and DSE, the sequence spanning nt 2273–2425 shows a high degree of homology when comparing different species (Fig. 5C), whereas the remaining 3′ UTR is poorly conserved. Computational folding under different parameters consistently suggests five stem-loop structures for the TSE (Fig. 6A; note that the 5′-4-base stem does not form regularly). The two 5′ truncations that abolish translational silencing, deleting nucleotides upstream of nt 2311 and 2333 (Fig. 5, A and B), would destroy the first structure (SL1), and the first two structures (SL1 and SL2), respectively. The 3′ truncation deleting nucleotides downstream of nt 2403 would destroy SL5. Indirect evidence supporting the importance of the putative secondary structure for TSE function can be derived from comparing the human and murine IκBz mRNA transcripts. The latter provides a “natural mutant” in which 11 positions are changed in the region corresponding to the TSE of the human counterpart (Fig. 6A). Seven of these changes affect unstructured regions, and four lie within stem structures. However, three of those result in changes of g-c to g-u pairing (SL5), respectively. Only one mutation extends a bulge in SL1. This “mutant” is still perfectly active not only in destabilization (supplemental Fig. S5) but also in translational silencing (Fig. 6B). It thus appears that mutations that preserve the secondary structure of this region leave its function intact.

In further experiments investigating the importance of secondary structure for the control of translation and stability, mutations were introduced in the third stem-loop (SL3), which still is contained in the DSE (nt 2333–2425). The SL3a mutation, which impairs the stem structure, strongly interfered with translational silencing (Fig. 6C). Mutation SL3b, which also impairs the stem structure, had an even stronger effect. A combination of both mutations (SL3c) restores the stem structure while altering the sequence. Importantly, this mutation did not significantly affect the translational silencing activity of the respective insert (Fig. 6C), supporting the functional importance of the stem-loop structure.

In sharp contrast to the consequences of structure-impairing mutations for translation as shown in Fig. 6C, the destabilizing activity was not affected (Fig. 6D). This confirms our observa-
IL-1-induced Post-transcriptional Control

A. 

B. 

C. 

D. 

E. 

fold suppression of luciferase activity

insertion: (-) 2273-2479 2333-2479 2311-2380

translational silencing: (-) + - -
destabilization: (-) + + -
tion above (Fig. 5) that sequence requirements for the translational regulation and control of degradation differ. The results further suggest that the stem-loop structure is not essential for destabilization. It has to be noted that the stem-loop structure is hypothetical so far and needs to be analyzed experimentally in future studies. Irrespective of this, the data above show that the effects on translation and degradation differ in their sequence requirements, with destabilization depending on a much shorter element and possibly more resistant to minor changes in the sequence.

The selective loss of translational silencing activity for some of the fragments in the presence of sustained destabilizing activity enabled us to perform an initial experiment for determining the relative contributions of both parameters for protein expression. The luciferase reporter activity in cells expressing luciferase with the complete TSE inserted (nt 2273–2479) was suppressed almost 40-fold (Fig. 6E), confirming the results obtained for insertion of the complete 3′ UTR (Table 2). Suppression was reduced to about 8-fold in response to IL-1. Luciferase activity generated by an mRNA with a 5′-truncated TSE (nt 2333–2479), which lacks translational silencing but still contains the complete DSE (see Fig. 5), is only suppressed about 3-fold compared with luciferase mRNA without insertion (Fig. 6E). No significant suppression was observed with a fragment of the TSE that was truncated on both ends and lacked both translational silencing and destabilizing activity. The results indicate that translational control by the IκBε TSE has a much stronger impact on protein expression than control of mRNA degradation.

**Translational Silencing and Destabilizing Functions Are Associated with Differential Protein Binding**—A radiolabeled RNA probe spanning the complete TSE formed four major UV-cross-linked complexes when incubated with cytoplasmic extract from HeLa cells (Fig. 7A, lane 2). The labeled RNA was displaced by excess unlabeled TSE RNA but not by irrelevant RNA, indicating selective protein-RNA interaction. Reduced formation of complex 3 was observed with proteins from IL-1-treated cells (Fig. 7A, lane 9). A 5′-truncated RNA fragment that lacks translational silencing activity but contains the complete DSE (nt 2311–2479) formed complex 2 less effectively (Fig. 7B, compare lanes 5 and 6), whereas intensities of the other complexes were preserved. Even stronger decrease of complex 2 was observed with the SL3a mutant of the TSE (lane 7), which exhibited impaired translational silencing but maintained destabilization (see Fig. 6, C and D). On the other hand, a 3′ truncation that abolished translational silencing and prolonged RNA half-life (nt 2273–2403) strongly impaired formation of both complex 2 and 3 and reduced formation of complex 4 (Fig. 7B, lane 8). Although these results are compatible with involvement of a complex 2 protein in translational silencing and of a complex 3 protein in destabilization, further work is required to identify these proteins and their functional roles.

**Evidence for Cap-dependent, Poly(A) Tail-independent Function of the TSE**—To find out if translational silencing by the IκBε TSE is cap-dependent or not, a bicistronic vector was used that contains the Renilla luciferase open reading frame (ORF), 3′-flanked by the polio virus IRES and the second ORF, encoding firefly luciferase (Fig. 8A, scheme). In cells transfected with this vector carrying the IκBε TSE inserted 3′ of firefly luciferase, the ratio of firefly luciferase activity to mRNA was not changed significantly, as compared with the vector without TSE insertion (Fig. 8A, black bars). Furthermore, IL-1 did not affect expression, irrespective of the presence of the TSE. This indicates that the TSE does not silence cap-independent translation as initiated by the polio virus IRES. Of note, Renilla luciferase expression by the first ORF (which roughly equals that of firefly luciferase, in agreement with Ref. 32) (see Fig. 3E of Ref. 32) also did not differ significantly (Fig. 8A). While the position of the TSE is close to the stop codon in IκBε mRNA as well as to the firefly luciferase stop codon in the bicistronic vector, it is distant from the Renilla ORF. The lack of suppression of this ORF might indicate that a stop-proximal location is important for TSE function. Indeed, insertion of the TSE directly 3′ of the Renilla ORF, whose translation is cap-dependent, suppressed its activity (Fig. 8B). IL-1, which had no consistent effect on expression from the vector without IκBε TSE, partly reversed this effect as expected (6.0- and 3.7-fold increase).

Evidence has been presented for binding of elf4G to polio virus IRES, recruitment of elf4A, and attachment of the 43 S preinitiation complex (see Ref. 42 and references therein). Resistance of initiation by this IRES to TSE-mediated suppression suggests that the latter affects an early step in initiation, at or before elf4G function. elf4G interacts with the cap-binding protein elf4E and the poly(A)-binding protein PABP. To test the importance of PABP and the poly(A)-tail for TSE function, a β-globin-IκBε reporter mRNA was expressed in which the polyadenylation signal was replaced by a histone stem-loop (30, 31). In histone mRNAs, the stem-loop replaces the poly(A)-tail and associates with elf4G through SLBP (stem-loop-binding protein) and its interacting partner SLIP (43). As shown in Fig. 8C, the β-globin-IκBε reporter mRNA containing the terminal histone stem-loop behaved like its polyadenylated counterpart; it was associated with non-polysomal fractions under unstimulated conditions and shifted to polysome fractions upon exposure of the cells to IL-1. The TSE-dependent silencing mechanism according to this result does not target PABP or the poly(A) tail.
Ribosome association of an mRNA is considered as a general measure of its translational activity (44, 45). The combination of analyzing ribosome association in sucrose gradients and quantifying mRNAs on microarrays has been successfully used to obtain genome-wide information on translationally regulated mRNAs (44, 46, 47). Taking this approach, we have identified a small group of mRNAs that exhibit increased ribosome association in response to IL-1 (Table 1, Fig. 1, and supplemental Fig. S1).

For IκBζ mRNA, the region mediating this effect could be localized to a highly conserved TSE encompassing nt 2273–2425 of its 3′ UTR. No obvious homology was observed when comparing the TSE with 3′ UTR sequences of the other transcripts translationally controlled by IL-1. In the murine IκBζ transcript, the region corresponding to the TSE has been reported to mediate post-transcriptional control; however, the contribution of mRNA stability versus translation was not examined (48). Within the human TSE, nt 2333–2425 contain a DSE that mediates rapid degradation under basal conditions and stabilization in response to IL-1 (Fig. 5). Thus, partly overlapping RNA elements mediate control of RNA stability and translation, the latter of which appears much more effective under the conditions applied here (Fig. 6E). The DSE contains only one AUUUA motif typical for AREs, and the neighboring sequences do not indicate a classical ARE. The function of the DSE could be distinguished from that of the TSE, based on several deletions and mutations that left destabilization unaffected but abolished translational silencing. Thus, translational silencing of the IκBζ mRNA is not a prerequisite of rapid degradation. This argues against a mechanism controlling IκBζ mRNA in which a translational silencing protein recruits a destabilizing protein as recently reported for TIA1 and KSRP (49). We cannot exclude the reverse situation, that a destabilizing mRNA-interacting protein recruits a translational silencer. The differential loss of binding of proteins to the TSE concomitant to selective loss of function upon truncation/mutation supports the involvement of functionally different proteins in TSE and DSE activity. Further studies are under way to clarify this point.

IL-1 appears to activate translation of IκBζ by impairing the function of a strong silencing element that minimizes synthesis of IκBζ protein in unstimulated cells. This might be a way to prevent deleterious consequences of IκBζ function as reflected by induction of apoptosis when overexpressed (16, 17). How IL-1 interferes with the silencing mechanism remains to be elucidated. Our information on the mechanism of silencing itself is
limited as well. Initiation is considered the rate-limiting step in translation, and often it is this part of the process and the factors involved in it that are targeted by regulatory mechanisms (12). Important for efficient initiation of translation is the interaction of the cap-binding protein eIF4E with the scaffolding protein eIF4G that also binds PABP (poly(A)-binding protein). All three proteins can be targets of inhibitory factors. PABP facilitates translation by stimulating recruitment of 40 S and joining of 60 S ribosomal subunits (52). Its function can be inhibited (e.g. by the PABP-interacting protein PAIP2) (53). Silencing of Drosophila msl-2 mRNA via its 3’S/11032 UTR is exerted by binding of SXL, which recruits UNR, a protein that in turn interacts with

FIGURE 8. Resistance of IRES-dependent translation and sensitivity of histone stem-loop-containing mRNA to TSE function. A, HeLa cells were transfected with a bicistronic vector (see scheme) without or with the IkBζ TSE inserted 3’ of the firefly luciferase ORF. The ratio of luciferase activity to mRNA for Renilla (Rluc) or firefly luciferase (Fluc) was calculated for cells incubated without or with IL-1α for 4 h as indicated and is presented as -fold change compared with the value for vector without insertion and without IL-1 (mean ± S.D., n = 4). B, Renilla luciferase activity/RNA ratios obtained for plasmids without or with the TSE inserted directly 3’ of the Renilla ORF are presented as -fold change compared with plasmid without TSE and no IL-1 treatment (mean ± S.D., n = 4). C, polysome profiles of β-globin mRNA containing the IkBζ TSE and a 3’-terminal histone stem-loop and of endogenous GAPDH mRNA as a control were determined in cells incubated without (con) or with IL-1α (IL-1) for 1 h. One of two experiments with similar results is shown.
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PABP (54). A mechanism directly targeting PABP is unlikely to explain silencing by the IκBζ TSE because its function and sensitivity to IL-1 are maintained in a transcript that harbors a histone stem-loop instead of a poly(A) tail at its 3′ end (Fig. 8C). Histone stem-loops take part in circularizing mRNAs by interacting with eIF4G through SLBP and SLIP instead of PABP (43).

Our observation that the IκBζ TSE does not silence poliovirus IRES-dependent translation (Fig. 8A) argues against the involvement of mechanisms like that inhibiting translation of 15-lipoxygenase during early erythroid differentiation at a late step in initiation, recruitment of the 60 S ribosomal subunit (55, 56). Because the poliovirus IRES binds specifically to eIF4G (42), our result is in accordance with a cap-dependent mechanism, affecting an early step in translation like eIF4E binding or its interaction with eIF4G. A prominent example for this type of translational regulation is the masking of eIF4E by Maskin, which associates with the CPEB protein that binds the cytoplasmic polyadenylation element of maternal mRNAs (57), a crucial step in the regulation of translation of maternal mRNAs during oogenesis and early development. Similarly, during development of the anterior-posterior axis in Drosophila, the Smaug protein binds nanos mRNA and represses its translation via a block in eIF4G recruitment by the Smaug-interacting protein Cup, which, like Maskin, acts as an eIF4E-binding protein (58).

Initial attempts to identify the signal transduction pathway involved in IL-1-induced translational activation and stabilization argue against involvement of the p38 MAPK pathway, which is known to cause stabilization of numerous ARE-containing mRNAs (e.g. see Ref. 59). The lack of effect when expressing the MAPK kinase kinase MEKK1 (supplemental Fig. S3B) confirms this notion and suggests that JNK and NF-κB pathways, which are strongly activated as well, are also dispensable. Inhibitors of p38 MAPK, JNK, PI3K, and MKK1 did not have a significant effect on translational activation by IL-1 (not shown). Because IRAK1 and -2, which function more upstream in IL-1 signaling, appear to be involved in translational activation (Fig. 4, B and C), the relevant signaling pathway apparently diverges downstream of IRAKs. This is reminiscent of IL-1-induced signaling that causes stabilization of certain mRNAs (e.g. Gro3) independently of p38 MAPK by an as yet unidentified pathway (10, 41). Interestingly, IL-17 has been found to trigger stabilization of these mRNAs as well (60, 61). In support of a common signaling pathway involved in stabilization and the translational activation reported here, IL-17, like IL-1, alleviates suppression of luciferase activity by the IκBζ TSE (not shown).

Selective enhancement of translation of a distinct set of mRNAs in response to IL-1 is likely to contribute to the changes in gene expression induced by this proinflammatory cytokine. There is no apparent sequence homology among the members of this group that would explain their common regulation by IL-1. However, it is noteworthy that two of the mRNAs affected by this type of regulation encode members of the CCCH-type zinc finger proteins, and two others encode members of the IκB family. IκBζ has been shown to support expression of a set of genes, including that of IL-6 (15, 18), whereas IκBδ counteracts IL-6 expression (62, 63). IL-6 mRNA itself is among the transcripts with increased ribosome association following IL-1 exposure. The CCCH zinc finger protein ZC3H12A, whose mRNA is also affected by IL-1, has been reported to suppress expression of several inflammatory proteins, including IL-6, and NF-κB-dependent luciferase expression (64) and recently to be involved in degradation of IL-6 mRNA (65). Cells of mice deficient in ZC3H12A show increased IL-6 expression (65). Thus, in addition to IL-6 itself, three other mRNAs are translationally activated that modify IL-6 expression. This suggests that translational activation by IL-1 has a profound influence on the course of IL-6 formation in inflammatory processes.

Like ZC3H12A, RC3H1 (also named roquin) is a CCCH-type zinc-finger protein. It contains a RING finger domain typical for ubiquitin ligases and was reported to limit expression of the T cell co-stimulatory receptor ICOS by promoting degradation of its mRNA (66). Mutation of the Rc3h1 gene can lead to autoimmunity (67).

It has to be noted that due to technical constraints (e.g. one time point of IL-1 induction, a selection of fractions, selected conditions of separation (time/speed of centrifugation, density profile of the gradient)), additional mRNA targets might have been missed. Because some of the mRNAs strongly induced by IL-1 are expressed in non-stimulated cells only at very low levels, hampering their detection on oligonucleotide arrays, important additional mRNA candidates for translational activation by IL-1 could not be evaluated (including, for example, IL-1α itself and IL-1β, which was suggested to be translationally controlled) (68, 69).

Several examples of translational control of inflammatory gene expression have been reported (70, 71). Activation of translation of negative regulators by IL-1 (Table 1) may achieve the same goal, eventual termination of the response, as IFN-γ-induced inhibition of translation; IFN-γ, which itself is translationally controlled by a PKR-activating sequence spanning part of its 5′ UTR and coding sequences (72, 73), restricts translation of some of its target genes through a heteromeric complex assembled at the target mRNAs (74).

We speculate that IL-1 activates a translational control mechanism that modulates expression of a group of genes, including IL-6, by increasing translation of their RNAs directly or through enhanced translation of factors controlling their transcription and mRNA stability. Identifying the signaling mechanism responsible for translational activation by IL-1 will be a crucial step in designing experiments to evaluate the impact of this control mechanism in a more physiological setting.

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