c-jun N-terminal kinase is involved in AUUUA-mediated interleukin-3 mRNA turnover in mast cells

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Whereas signalling pathways involved in transcriptional control have been studied extensively, the pathways regulating mRNA turnover remain poorly understood. We are interested in the role of mRNA stability in cell activation and oncogenesis using PB-3c mast cells as a model system. In these cells the short-lived interleukin-3 (IL-3) mRNA is stabilized by ionomycin treatment and following oncogenesis. To identify the signalling pathways involved in these mechanisms, we analysed the effect of different kinase inhibitors. SB202190 and wortmannin were shown to antagonize ionomycin-induced IL-3 mRNA stabilization in PB-3c cells in the presence of actinomycin D, and this effect coincided with their ability to inhibit c-jun N-terminal kinase (JNK) activation by ionomycin. Moreover, transfection of activated MEKK1 amplified ionomycin-induced IL-3 mRNA expression at the post-transcriptional level, and a dominant-negative mutant of JNK counteracted mRNA stabilization by ionomycin. Taken together, these data indicate that JNK is involved in the regulation of IL-3 mRNA turnover in mast cells. In addition, transfection experiments revealed that the *cis*-acting AU-rich element in the 3' untranslated region of IL-3 mRNA is necessary and sufficient to confer JNK-dependent mRNA stabilization in response to cell activation.

Keywords: ARE/IL-3/JNK/mast cells/mRNA turnover

Introduction

Turnover of mRNA is an important mechanism for the regulation of gene expression in organisms ranging from bacteria to mammals. Accordingly, mRNA levels can fluctuate many-fold following a change in mRNA half-life without altering the transcription rate (Ross, 1995, 1996). Regulation of mRNA half-life can influence normal cell growth, differentiation and oncogenesis. Expression of growth factors, cytokines and proto-oncogenes may be regulated in this way (Shaw and Kamen, 1986; Thorens *et al.*, 1987; Wodnar-Filipowicz and Moroni, 1990; Chen and Shyu, 1995).

We are interested in the role of mRNA stability in cell activation and oncogenesis using normal, interleukin-3 (IL-3)-dependent, PB-3c mast cells as a model system. These cells produce IL-3 and other cytokines upon activa-

tion by extracellular signals (Wodnar-Filipowicz *et al.*, 1989; Wodnar-Filipowicz and Moroni, 1990). The corresponding transcripts are short-lived with half-lives of ~30 min, but can be stabilized by treatment with Ca²⁺ ionophores. Furthermore, stabilization of IL-3 mRNA was also found to play a role in autocrine, IL-3-producing tumours derived from PB-3c cells following v-H-ras expression. Most of these tumours carry a defect in the IL-3 mRNA decay mechanism and display IL-3 mRNA half-lives of several hours with concomitant activation of the autocrine loop (Nair *et al.*, 1989, 1992; Hirsch *et al.*, 1993, 1995). The underlying mechanism of this defect appears to occur in *trans*, as cell fusion of autocrine tumours with normal precursor cells restores physiological rapid decay of IL-3 mRNA. Reversion of cell growth to IL-3 dependency and partial tumour suppression is also observed (Diamantis *et al.*, 1989; Hirsch *et al.*, 1995).

IL-3 transcripts contain within their 3' untranslated region (3' UTR) an AU-rich element (ARE), a characteristic of many short-lived mRNAs (Shaw and Kamen, 1986; Chen and Shyu, 1995). The ARE of IL-3 consists of eight canonical AUUUA motifs (Campbell *et al.*, 1985) that generate two adjacent consensus nonamers [UUAUUUA(U/A) (U/A)] known to direct rapid mRNA degradation (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995). A mutational analysis of the ARE revealed that both nonamers had to be mutagenized in order to abrogate the decay function (Stoecklin *et al.*, 1994). Furthermore, hybrid mRNA between stable β -globin and the ARE from IL-3 had a short half-life but could be stabilized by Ca²⁺ ionophores. Taken together, these results suggested that the ARE mediates decay in a Ca²⁺-regulated fashion.

Whereas AREs are now well defined as *cis*-elements (Chen and Shyu, 1994, 1995; Stoecklin *et al.*, 1994; Chen *et al.*, 1995; Xu *et al.*, 1997), little is known about the signalling pathways regulating ARE-mediated mRNA turnover, in contrast to today's understanding of how signalling pathways regulate transcription. Recently, a number of selective inhibitors for known signalling pathways has become available. The immunosuppressant cyclosporin A (CsA), upon forming a complex with cyclophilin A or B, binds to and inhibits the Ca²⁺- and calmodulin-dependent phosphatase calcineurin (Liu *et al.*, 1991). FK506, complexed to FKBP12, similarly inhibits calcineurin (Liu *et al.*, 1991). Rapamycin, a third immunosuppressant, shares the binding protein FKBP12, but the complex inhibits another target, FRAP or RAFT (Brown *et al.*, 1994; Sabatini *et al.*, 1994), the mammalian homologue of yeast TORs (target of rapamycin), phosphatidylinositol kinase homologues (Kunz *et al.*, 1993). The antibiotic wortmannin has been shown to specifically inhibit phosphatidylinositol 3-kinase (PI3-K) by irreversibly binding to its catalytic subunit p110 (Yano *et al.*, 1993; Thelen *et al.*, 1994). The methylxanthine cAMP

phosphodiesterase inhibitor SQ20006 (Chasin *et al.*, 1972) has been reported, like rapamycin and wortmannin, to inhibit p70^{s6k} activation indirectly (Kuo *et al.*, 1992; Chung *et al.*, 1994; Han *et al.*, 1995). SB202190 belongs to a novel class of pyridinyl imidazoles that were developed as inhibitors of the lipopolysaccharide-induced synthesis of IL-1 and tumour necrosis factor (TNF) from preformed mRNA in monocytes (Young *et al.*, 1993), and whose target was then identified as CSBP1 and CSBP2, human homologues of p38 mitogen-activated protein kinase (MAPK) (Lee *et al.*, 1994). PD98059 was the first compound reported to inhibit MAPK/ERK-activating enzyme MEK and, consequently, the extracellular signal-regulated kinase (ERK) pathway (Dudley *et al.*, 1995; Levitzki, 1996). These selective signalling inhibitors provide potential tools for evaluating the roles of relevant signalling pathways in a biological response of interest. Indeed, the specific p38 MAPK inhibitor SB203580 has been successfully employed in demonstrating the role of p38 MAPK in regulating IL-6 synthesis in response to TNF (Beyaert *et al.*, 1996), and in regulation of CREB and ATF-1 by FGF (Tan *et al.*, 1996).

Using these inhibitors, we demonstrate that the *c-jun* N-terminal kinase (JNK) is involved in the stabilization of IL-3 mRNA by Ca²⁺. This was confirmed by a transfection approach using a dominant-negative mutant of JNK which antagonized mRNA stabilization by ionomycin in the presence of the transcription inhibitor actinomycin D (actD). Moreover, in this paper we show that the ARE in the 3' UTR is the *cis*-element through which the JNK pathway exerts its regulatory effect on mRNA turnover.

Results

Cyclosporin A, SB202190 and wortmannin inhibit ionomycin-induced IL-3 mRNA expression at the post-transcriptional level

To identify signalling pathways that regulate IL-3 mRNA turnover, we studied the effect of selective signalling inhibitors on ionomycin-induced IL-3 mRNA expression in PB-3c-15 mast cells. As shown in Figure 1A, treatment of these cells with CsA, FK506, wortmannin or SB202190 inhibited IL-3 mRNA induction (compare lane 3, 4, 6 and 8 with lane 2), whereas no effect was observed when cells were treated with rapamycin, SQ20006 or PD98059 (lanes 5, 7 and 9).

Our previous work demonstrated that IL-3 mRNA can be stabilized in mast cells by treatment with Ca²⁺ ionophores or with the PKC activator phorbol 12-myristate 13-acetate (PMA) (Wodnar-Filipowicz and Moroni, 1990; Hahn and Moroni, 1994). To demonstrate that the inhibitory effects of these drugs are at the post-transcriptional level, we performed a decay assay in the presence of actD. Taking into account that both CsA and FK506 exert their effects through the calcineurin pathway, only CsA, together with SB202190 and wortmannin were used for further analysis. Consistent with previous data, addition of actD resulted in rapid decay of IL-3 transcripts (Figure 1B, lanes 1–4), and treatment of cells with ionomycin stabilized the transcripts (Figure 1B, lanes 5–8). All three drugs inhibited ionomycin-induced IL-3 mRNA stabilization in the presence of actD (Figure 1B, lanes 9–20). Quantifica-

tion of these results revealed that the drugs exert various degrees of inhibition, although none gives a 100% effect (Figure 1C). We concluded that CsA, SB202190 and wortmannin inhibit ionomycin-induced IL-3 mRNA stabilization.

Stress MAPKs are the common effectors targeted by CsA, SB202190 and wortmannin

As CsA, SB202190 and wortmannin have different intracellular targets, we asked whether three different signalling pathways control IL-3 mRNA stabilization, or whether the three drugs target a common downstream effector. Indeed, JNK has been reported to be sensitive to these drugs. In Jurkat T-cells, CsA inhibited JNK activation by the tumour promoter TPA and Ca²⁺ ionophore treatment (Su *et al.*, 1994). In HeLa cells, evidence was presented that PI3-K activity plays a role in EGF-induced JNK activation in a wortmannin-sensitive fashion (Logan *et al.*, 1997). Moreover, FcεR-mediated JNK activation in mast cells was also significantly suppressed by wortmannin (Ishizuka *et al.*, 1997). Although p38 MAPK was identified as a specific target of the pyridinyl imidazoles including SB202190 (Lee *et al.*, 1994), recent studies indicate that they block activation of the JNK pathway as efficiently as p38 MAPK (M.Karin, personal communication). JNK and p38 MAPK are closely related members of MAPK subfamilies with respect to both stimulus activation specificity and biological function (Minden *et al.*, 1994; Xia *et al.*, 1995; Graves *et al.*, 1996); therefore we analysed the effects of CsA, SB202190 and wortmannin on the activation of both JNK and p38 MAPK by ionomycin treatment of PB-3c-15 cells. The experiments were carried out in the absence or presence of actD, the results from both sets of experiments were consistent. As shown in Figure 2, ionomycin activated both JNK and p38 MAPK (Figure 2A–C, lanes 2 and 7). Treatment of cells with SB202190 or wortmannin prior to ionomycin induction significantly reduced JNK activation (Figure 2A, lanes 4, 5, 9 and 10). In contrast to the inhibitory effect of CsA on TPA/Ca²⁺ ionophore-induced JNK activation reported in Jurkat cells (Su *et al.*, 1994), no significant effect on ionomycin-induced JNK activation was observed when mast cells were pre-treated with CsA (Figure 2A, lanes 3 and 8). CsA, however, showed an inhibitory effect on p38 MAPK activation (Figure 2B, lanes 3 and 8), whereas SB202190 or wortmannin did not (Figure 2B, lanes 4, 5, 9 and 10). The fact that p38 MAPK inhibitor, SB202190, did not show a detectable effect on p38 MAPK could be explained by the mode of drug action and the character of the kinase assay. SB202190 does not inhibit the activation step, but is inhibitory by binding directly to the ATP binding site of p38 MAPK (Young *et al.*, 1997). During immunoprecipitation required for the assay, the drug dissociates from p38 MAPK; thus no inhibitory effect could be observed in an *in vitro* kinase assay (Figure 2B). The drug did, however, inhibit the p38 MAPK cascade *in vivo* when assayed for the phosphorylation status of CREB (Figure 2C), a downstream component of the p38 MAPK pathway (Tan *et al.*, 1996). These data show that stress MAPKs are common downstream effectors of CsA, SB202190 and wortmannin. SB202190 suppressed ionomycin-induced activation of both p38 MAPK and

Fig. 1. Cyclosporin A, SB202190 and wortmannin inhibit ionomycin-induced IL-3 mRNA expression in PB-3c-15 mast cells at the post-transcriptional level. **(A)** Cells were either untreated (lane 1) or treated with 2 μ M ionomycin (iono) for 3 h in the absence (lane 2) or presence of various drugs as indicated: 500 ng/ml CsA (lane 3); 20 ng/ml FK506 (FK, lane 4); 20 ng/ml rapamycin (Rap, lane 5); 200 nM wortmannin (WM, lane 6); 500 nM SQ20006 (SQ, lane 7); 20 μ M SB202190 (SB, lane 8) and 50 μ M PD98059 (PD, lane 9). **(B)** Decay assay of IL-3 mRNA in the presence of CsA, SB202190 and wortmannin. Cells were pretreated with 2 μ M ionomycin for 3 h (-3 h to 0), washed, and then incubated further under conditions indicated at the bottom of the figure. Actinomycin D (ActD, 5 μ g/ml) was added at time 0 and RNA levels were determined after 0, 0.5, 1 and 2 h. Note that after treatment with ionomycin for 3 h, cells were washed and resuspended in ionomycin-free medium prior to the addition of actD, ionomycin or various drugs. The concentration of drugs was the same as used in (A). **(C)** Quantification of the signals shown in (B) by PhosphorImager, where the actin-normalized values at time 0 were taken as 100%.

JNK pathways, whereas CsA was inhibitory only for the p38 MAPK, and wortmannin only for the JNK pathway.

Activated MEKK1 amplified, but dominant-negative JNK suppressed ionomycin-induced IL-3 mRNA expression at the post-transcriptional level

To evaluate the potential role of JNK or p38 MAPK in IL-3 mRNA stabilization, PB-3c-15 cells were stably transfected with: (i) MEKK1 Δ , a constitutive activator of JNK, which at high levels of expression also activates p38 MAPK (Minden *et al.*, 1994; Yan *et al.*, 1994; Lin *et al.*, 1995); (ii) MEK6-DD, a constitutive activator of p38 MAPK (Stein *et al.*, 1996); (iii) JNK-APF, a dominant-negative mutant of JNK (Gupta *et al.*, 1995); and (iv) p38-AGF, a dominant-negative mutant of p38 MAPK. JNK assays and detection of the phosphorylation status of the p38 MAPK downstream component CREB were performed to monitor the functional expression of the constructs on the two pathways. As shown in Figure 3A, panels a–c, transfection of MEKK1 Δ alone did not activate the JNK or p38 MAPK pathway, but amplified the activation of JNK by ionomycin (Figure 3A, panels a and b) without a detectable effect on ionomycin-induced phosphorylation of CREB (Figure 3A, panel c). Moreover, JNK-APF suppressed ionomycin-induced JNK activation. However, none of the constructs transfected revealed

a significant effect on the basal or ionomycin-induced phosphorylation of CREB (Figure 3A, panel c). Taken together, these data indicate that MEKK1 Δ and JNK-APF are functionally expressed, in contrast to MEK6-DD or p38-AGF.

Consistent with their functional expression, Northern blot analysis revealed that MEKK1 Δ amplified, and JNK-APF impaired ionomycin-induced IL-3 mRNA expression, whereas no effect was seen with MEK6-DD or p38-AGF (Figure 3B). As JNK is known to be involved in transcriptional control, we next carried out mRNA decay and nuclear run-on assays to evaluate whether the observed effects of MEKK1 Δ or JNK-APF on IL-3 mRNA expression occur at the transcriptional or post-transcriptional level. Figure 3C shows the decay assays performed in the presence of actD. Again, as shown in Figure 3B, the presence of MEKK1 Δ amplified ionomycin induction (Figure 3C, left panel, compare lane 6 with lane 1). However, when ionomycin was washed out and actD was added in the absence of the inducer, IL-3 mRNA decayed at similar rates in both PB-3c-15 and PB-3c-15/MEKK1 Δ lines. This correlated with the fact that JNK was not activated in PB-3c-15/MEKK1 Δ in the absence of ionomycin, and it appears that IL-3 mRNA cannot be stabilized in PB-3c-15/MEKK1 Δ in the absence of ionomycin. To confirm that the effect of MEKK1 Δ is post-transcriptional,

Fig. 2. Stress MAPKs are common downstream effectors of cyclosporin A, SB202190 and wortmannin. Cells were cultured as described in Figure 1A. After pre-treatment with CsA, SB202190 or wortmannin in the presence or absence of actD for 20 min as indicated in the figure, the cells were untreated or stimulated with ionomycin for 15 min. Lysates were then prepared and 100 µg lysate was subjected to *in vitro* JNK (A) or p38 kinase (B) assay using GST-jun (1–79) or GST-ATF2 (1–254) as substrate, respectively. CREB phosphorylation was analysed by Western blot using antibodies specific for phosphorylated Ser133 of CREB (P-CREB) or phosphorylated Ser63 of ATF1 (P-ATF1) (C). CREB expression was analysed by Western blot using antibodies that recognize CREB regardless of their phosphorylation status (D).

nuclear run-on assays were performed using nuclei prepared from cells stimulated with ionomycin. The data shown in Figure 3D revealed that MEKK1Δ did not detectably affect IL-3 transcription, indicating that the role of JNK activation is at the post-transcriptional level. Confirming evidence for this proposition was provided when the effect of JNK-APF, a dominant-negative mutant of JNK, was examined for its effect on IL-3 mRNA stabilization. Whereas ionomycin stabilized the mRNA in PB-3c-15 cells in the presence of actD, this effect was not observed in PB-3c-15/JNK-APF cells under the same experimental conditions (Figure 3C, right panel). This suggests that the JNK signalling pathway is required for the stabilizing effect of ionomycin. Moreover, JNK-APF did not significantly affect IL-3 transcription in nuclear run-on assays (Figure 3D). Taken together, these data, along with the inhibitor data (Figure 1B and C), demonstrate that activation of the JNK pathway is required for ionomycin-induced IL-3 mRNA stabilization in mast cells.

The ARE in the 3' UTR is the *cis*-element required for JNK-dependent stabilization

The ARE in the 3' UTR of IL-3 mRNA is both necessary and sufficient for directing rapid decay of IL-3 transcripts (Stoecklin *et al.*, 1994). We therefore examined whether the ARE is also the *cis*-element regulated by the JNK pathway. We consequently took advantage of PB-3c-15 cells stably transfected with IL-3 genes carrying different mutations within the ARE, and thus displaying various half-lives (Stoecklin *et al.*, 1994). Figure 4A schematically displays the localization of the mutated AUUUA motifs within the ARE, together with the previously determined

half-lives of the respective transcripts (Stoecklin *et al.*, 1994). RNase protection assays (Figure 4B) revealed the effect of SB202190 on stabilization with the various IL-3 transgenes compared with the endogenous IL-3 mRNA. Note that the IL-3 transgenes are tagged by two adjacent silent point mutations in exon 3, so that the endogenous transcripts may be distinguished from the exogenous ones (Nair *et al.*, 1994). Cells were first treated with ionomycin for 3 h, followed by decay assays in the presence of actD. After washing out ionomycin, cells were treated with actD alone (lanes 1), or in combination with ionomycin in the absence (lanes 2) or presence (lanes 3) of SB202190 for a further 2 h. This allowed the decay of the transcripts (lanes 1), stabilization of the transcripts by ionomycin (lanes 2), or the effect of SB202190 on ionomycin-induced stabilization (lanes 3), respectively, to be revealed. In agreement with previous results (Stoecklin *et al.*, 1994), all the transcripts with a half-life of ~30 min [including wild-type (wt), 1a, 1c, 1d, 2b and 3d] were degraded substantially after 2 h treatment with actD (lanes 1) but were stabilized by ionomycin (lanes 2). Transcripts with a half-life >3 h (3a, 6a and ΔAU) showed no difference between the two treatments. SB202190 inhibited ionomycin-induced stabilization of the labile transcripts, but not of the stable transcripts 3a, 6a and ΔAU (lanes 3). Note that the endogenous transcripts (shown below the top band of the figure) in these transfectants behaved like the short-lived wt transcripts. These data suggested that SB202190, a drug exploited here to monitor the activity of the JNK pathway, did not promote degradation via sequences outside the ARE.

We also examined whether the 5' UTR of IL-3 mRNA may also be a *cis*-element for JNK regulation and used a transgene in which the 27 nucleotides corresponding to the entire 5' UTR were removed. The wt and the deletion genes were transfected into PB-3c-15 cells, and stably transfected lines (PB-3c-15/M1h-IL3-wt, PB-3c-15/M1h-IL3-Δ5'UTR1 and PB-3c-15/M1h-IL3-Δ5'UTR2) were obtained following selection. All lines grew independently of added IL-3 and secreted the lymphokine. Also, both mutant lines behaved as the wt line with respect to mRNA decay and stabilization (data not shown). The sensitivity towards SB202190 was analysed in cells pretreated with actD, stimulated with ionomycin in the presence or absence of SB202190, as indicated in Figure 4C. The results revealed that transcripts containing the 5' UTR deletion behaved as wt transcripts and were sensitive to SB202190. These data imply that the 5' UTR of IL-3 mRNA is not necessary for JNK-regulated stabilization of IL-3 mRNA.

As a next step, we determined whether the 3' UTR of IL-3 would confer drug sensitivity to a heterologous transcript. A β-globin reporter construct with the ARE-containing 3' UTR of IL-3 was used for this purpose and compared with β-globin with its own 3' UTR. As shown in Figure 4D, β-globin mRNA was stable for >2 h and was insensitive to both SB202190 and wortmannin. The presence of an ARE from IL-3 conferred rapid decay and sensitivity to the two drugs. Similar results were obtained with a construct where the ARE was limited to six AUUUA motifs (data not shown). These data support the conclusion that ARE in the 3' UTR of IL-3 mRNA is the *cis*-element for JNK-dependent stabilization.

Fig. 3. A constitutively active MEKK1 (MEKK1 Δ) amplified, and a dominant-negative mutant of JNK impaired IL-3 mRNA expression in PB-3c-15 cells at the post-transcriptional level. PB-3c-15 mast cells were stably transfected with (i) MEKK1 Δ , a constitutive activator of JNK; (ii) MEK6-DD, a constitutive activator of p38; (iii) a dominant-negative mutant of JNK (JNK-APF); and (iv) a dominant-negative mutant of p38 (p38-AGF). (A) Functional expression of transfected constructs was examined by their effect on JNK activity (panels a and b) or phosphorylation of CREB (panels c and d). Cells were either untreated (lanes 1, 3, 5, 7 and 9) or treated with ionomycin for 15 min (lanes 2, 4, 6, 8 and 10). Extracts were subjected to *in vitro* JNK assay (panels a and b, where the former is a longer exposure of panel b). CREB phosphorylation (panel c) and CREB expression (panel d) were analysed by Western blot as described in Figure 2. (B) The effect of the introduced cDNAs on IL-3 mRNA expression was analysed by Northern blot. Cells were either untreated or treated with ionomycin for 3 h, followed by incubation for a further 2 h in the presence of actD, as indicated below the figure. (C) Decay assay of IL-3 mRNA as described in Figure 1B. The left panel shows the decay of IL-3 mRNA in the absence of ionomycin in PB-3c-15 and PB-3c-15/MEKK1 Δ cells. The right panel reveals the stabilization of IL-3 mRNA by ionomycin in PB-3c-15 and the lack of stabilization by ionomycin treatment in PB-3c-15/JNK-APF cells. In both cases, cells were pre-stimulated with ionomycin for 3 h and then washed out prior to the addition of actD in the absence (left panel) or presence (right panel) of ionomycin. Lanes 1 and 6 correspond to samples taken before washing. The lower panels show the quantification of the signals, the phosphorimage storage limit was not exceeded for lanes 11–15. (D) Nuclear run-on analysis of IL-3, actin and GM-CSF transcripts in PB-3c-15, PB-3c-15/MEKK1 Δ and PB-3c-15/JNK-APF lines. Nuclei were prepared from cells stimulated with ionomycin for 3 h.

A **B** **D** **C**

Fig. 4. ARE is the *cis*-element required for JNK-dependent IL-3 mRNA stabilization. (A) Scheme of the 3' UTR of the IL-3 gene containing an ARE with eight AUUUA motifs represented as boxes numbered I–VIII. The non-numbered box represents AUUUUA. Shown on top is the wt sequence, below the mutated alleles 1a, 1c, 1d, 2b, 3a, 3d, 6a and ΔAU, where an asterisk indicates a U→G mutation. The mRNA half-life of each construct as reported previously (Stoecklin *et al.*, 1994) is indicated at the right of the scheme in parentheses. (B) Effect of SB202190 on stabilization of wt and mutated transcripts. Cells were stimulated with ionomycin for 3 h, washed, and further incubated for 2 h in the presence of actD, with or without ionomycin and/or SB202190, as indicated. (C) Removal of the 5' UTR of IL-3 does not affect stabilization by ionomycin or destabilization by SB202190. Expression of transfected IL-3 genes without (M1h-IL3-wt) or with 5' UTR deletion (M1h-IL3-Δ5'UTR) was analysed by Northern blot. Transfected PB-3c-15 cells were pre-treated with actD for 30 min followed by the addition of ionomycin with or without SB202190 for another 2 h, still in the presence of actD. Note that pre-treatment with actD prior to stimulation abrogates induction of the endogenous transcripts allowing the detection of mRNA only from the transgenes. M1h-IL3-Δ5'UTR1 and M1h-IL3-Δ5'UTR2 represent two independently transfected lines. (D) The 3' UTR of IL-3 mRNA confers rapid decay and SB202190/wortmannin sensitivity to a reporter construct. Schematic representation of the β-globin reporter constructs are shown on top. LTR: the Moloney leukaemia virus long terminal repeat. The wide bars represent the open reading frame (ORF) of β-globin. The narrow bars represent the 5' UTR and 3' UTR, where 3' UTR on the right is from IL-3 and contains an ARE (solid box). Stably transfected PB-3c-15 cells were pre-treated with actD for 30 min followed by the addition of ionomycin with or without SB202190 or wortmannin for another 2 h, still in the presence of actD.

Discussion

The work reported here was designed to identify a link between the signal transduction pathway and IL-3 mRNA turnover. We provide evidence that JNK, a well-characterized signalling component of transcription, is also a regulator of IL-3 mRNA stability in mast cells. The corresponding *cis*-element is the ARE within the 3' UTR of IL-3 mRNA, which is both necessary and sufficient for directing the turnover of the transcripts and conferring JNK-dependent stabilization.

MAPKs are serine–threonine kinases that have important functions as mediators of cellular responses to a variety of extracellular stimuli (Robinson and Cobb, 1997). In mammalian cells, three major subfamilies of MAPKs have been identified, including the ERKs, JNK and p38 MAPK. ERKs are characteristically activated by various growth factors and phorbol esters, whereas JNK and p38 MAPK subfamilies are mediators of the pro-inflammatory cytokines TNF and IL-1, as well as stress stimuli such as UV-radiation, heat shock and hyperosmolarity (Robinson and Cobb, 1997). In PB-3c-15 cells, ionomycin activated JNK,

p38 MAPK (Figure 2) and ERK (data not shown). This pleiotropic activation may be mediated by Ca^{2+} /calmodulin-dependent kinase IV cascade as reported recently with PC-12 cells (Enslin *et al.*, 1996).

In the present study, selective signalling inhibitors were used to probe regulatory pathways that govern IL-3 mRNA turnover. Of the inhibitors tested, the calcineurin inhibitor CsA, the stress MAPKs inhibitor SB202190 and the PI3-K inhibitor wortmannin antagonized the stabilizing effect of ionomycin in the presence of actD (Figure 1). SB202190 and, at the same time, wortmannin inhibited ionomycin-induced JNK activation (Figure 2A), and SB202190 also inhibited the p38 MAPK cascade (Figure 2C). CsA, known to inhibit JNK in TPA/ Ca^{2+} ionophore-stimulated human Jurkat T-cells (Su *et al.*, 1994), did not show this activity in PB-3c-15 mast cells. In contrast, it inhibited p38 MAPK (Figure 2B and C). These data suggest that JNK and p38 MAPK, in addition to their role in transcriptional regulation, alone or in combination, also mediate mRNA stabilization.

To test the above proposition, we generated cell lines stably transfected with activators or dominant-negative mutants of JNK and p38 MAPK pathways. The upstream activator of JNK, MEKK1 Δ , amplified IL-3 mRNA induction by ionomycin (Figure 3B and C) to a degree which could not be explained by transcriptional activation, as indicated by nuclear run-on analysis (Figure 3D), arguing that JNK activation is involved in the stabilization of IL-3 mRNA. Importantly, JNK-APF, a dominant-negative mutant of JNK, antagonized IL-3 mRNA stabilization in the presence of actD (Figure 3C, right panel). These results indicate that the JNK pathway is essential for IL-3 mRNA stabilization; however, it may not be sufficient, since transfection of MEKK1 Δ neither resulted in constitutive elevated activity of JNK (Figure 3A, panels a and b) nor in stabilization of IL-3 mRNA in the absence of ionomycin (Figure 3B and C, left panel). This may reflect the fact that overexpression of MEKK1 Δ induces apoptosis through JNK (Minden *et al.*, 1994; Xia *et al.*, 1995; Verheij *et al.*, 1998). Only cells expressing very low levels of MEKK1 Δ may therefore be selected during stable transfection. Additional signals in parallel to JNK may be required for full stabilization. This is suggested by our data using CsA, which antagonizes stabilization (Figure 1) without inhibiting JNK activation (Figure 2A). This may be due to the requirement of an unknown pathway involving the CsA target calcineurin, or a downstream component of JNK may be sensitive to CsA. Note that CsA, along with SB202190, also inhibited the p38 MAPK cascade (Figure 2B–D). As neither the upstream activator of p38 MAPK, MEK6-DD, nor the dominant-negative mutant p38-AGF were functionally expressed (Figure 3A), the role of p38 MAPK is not yet clear.

In view of the fact that IL-3 mRNA is stabilized by induction of an activated N-ras gene in PB-3c cells as reported previously (Hahn *et al.*, 1991), it is noteworthy that the ras-dependent ERK pathway is not required for ionomycin-induced stabilization, as shown by using the MEK inhibitor PD98059. This drug did not affect IL-3 expression tested at 50 μM (Figure 1A), although it inhibited the activation of ERKs by ionomycin at the same concentration (data not shown). Whereas the destabilizing effect of SB202190 and wortmannin is explained by JNK

inhibition, the effect of wortmannin also indicates that JNK activation in PB-3c-15 cells is controlled by PI3-K, as proposed with HeLa cells (Logan *et al.*, 1997). The results with wortmannin (Figures 1 and 2) and activated N-ras (Hahn *et al.*, 1991) argue that activated p21^{ras} signals via PI3-K and rac to JNK (Rodriguez-Viciano *et al.*, 1994; Coso *et al.*, 1995; Nobes and Hall, 1995) which then, perhaps in cooperation with another pathway, leads to mRNA stabilization.

Our data suggest that the JNK pathway is involved in regulating an ARE-binding protein complex including an RNase. The *cis* target of this mechanism is the ARE present in the 3' UTR of IL-3 mRNA, which is both necessary and sufficient as indicated by the following evidence. (i) The labile hybrid transcripts containing the stable haemoglobin- β chain and the IL-3 3' UTR could be stabilized by ionomycin, and destabilized by SB202190 or wortmannin (Figure 4D). (ii) SB202190 destabilized transcripts containing wt AUUUA motifs and those with AUUUA mutations that do not affect half-life. In contrast, transcripts with stabilizing mutations were rendered unresponsive to both ionomycin and SB202190 (Figure 4B). This is best explained as proposed previously (Stoecklin *et al.*, 1994) by an ARE-binding regulatory protein that, when bound to RNA, leads to constitutive rapid transcript decay. Mutations in the consensus sequence of the ARE, or elevated Ca^{2+} leading to modification of the *trans*-acting regulator counteract the constitutive decay pathway by preventing the complex formation. The emerging model is that ARE-mediated mRNA turnover is controlled by an interplay of kinases and phosphatases regulating ARE-dependent *trans*-acting factors. This is also supported by a recent study with AUF1, an ARE-binding protein, indicating that the complex formation with GRO α mRNA and the consequent accelerated mRNA turnover are phosphorylation-dependent events sensitive to protein kinase inhibitors (Sirenko *et al.*, 1997). It will be interesting to see whether HuR (Ma *et al.*, 1996; Myer *et al.*, 1997), another ARE-binding protein recently shown to stabilize mRNA *in vivo* (Fan and Steitz, 1998; Peng *et al.*, 1998), is also regulated through phosphorylation.

If a *trans*-acting negative regulator of IL-3 mRNA turnover indeed mediates degradation by forming a complex with the ARE in a JNK-regulated fashion, mobility gel-shift assays with IL-3 RNA probes might reveal ionomycin-regulated RNA–protein complex formation. Preliminary experiments revealed the existence of ARE-binding proteins, but the complex formation was not altered by ionomycin treatment of cells (A. Wyss, unpublished data). One possibility is that ionomycin stabilizes mRNA by inactivating a ribonuclease in the RNA–protein complex rather than affecting complex formation between mRNA and the ARE-binding protein.

While this manuscript was in preparation, Chen *et al.* (1998) published similar information with IL-2 mRNA. In contrast to our data, the 3'-terminal ARE was not sufficient for JNK-mediated stabilization of IL-2 transcripts, but required additional 5'-terminal sequences. The reason why these two interleukins display different *cis* requirements for JNK-dependent mRNA stabilization remains to be shown.

We have now demonstrated a link between the JNK pathway and IL-3 mRNA turnover. A full understanding

of this aspect of post-transcriptional regulation requires the identification and cloning of the responsible *trans*-acting regulatory components including the ribonuclease.

Materials and methods

Materials

Reagents were purchased or obtained from the following sources: CsA and wortmannin from Sigma; actD and hygromycin B from Calbiochem; rapamycin from Sandoz; SQ20006 from Squib; PD98059, phospho-specific CREB (Ser383) antibodies and CREB antibodies were all from New England Biolabs. The puridiny imidazole SB202190 and polyclonal anti-p38 MAPK antibodies were kindly provided by Dr M.Karin. The recombinant GST-*c-jun* (1–79) and GST-ATF2 (1–254) fusion proteins were purified as described previously (Hibi *et al.*, 1993).

Cell culture and transfection

PB-3c-15 is a subclone of the murine IL-3-dependent PB-3c mast cell line (Nair *et al.*, 1992). PB-3c-15/Mxh-IL3-wt, -1a, -1c, -1d, -2b, -3a, -3d, -6a, and -ΔAU are PB-3c-15 cells transfected with IL-3 gene constructs mutated in the 3' UTR as described previously (Stoecklin *et al.*, 1994). PB-3c-15/β-globin, PB-3c-15/β-globin-ARE, PB-3c-15/M1h-IL3-wt, -Δ5'UTR1 and -Δ5'UTR2 are derived by electroporation of PB-3c-15 using the constructs pMxh-β-globin, pMxh-β-globin-ARE, pM1h-IL3-wt and pM1h-IL3-Δ5'UTR, respectively. PB-3c-15/MEKK1Δ, PB-3c-15/MEK6-DD, PB-3c-15/JNK-APF, and PB-3c-15/p38-AGF are stably transfected lines obtained by electroporation (Bio-Rad Gene Pulser) with 5 μg of pZH2-hygromycin plasmid together with 50 μg of SRα3-MEKKΔ, SRα3-MEK6DD, pCMV-JNK-APF or pCMV-p38-AGF plasmid, respectively. Selection was in 1 mg/ml hygromycin B (Calbiochem). All cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and conditioned medium from X63-mIL3 cells as source of IL-3 as described previously (Nair *et al.*, 1992).

Plasmid constructs

Construct pM1h-IL3-wt contains 4.3 kb *HindIII*–*SpeI* fragment of murine genomic IL-3 which includes 1.8 kb of 5' flanking sequence and 0.6 kb of 3' flanking sequence. The construct contains all of the 5'- and 3' flanking sequences necessary for ionomycin induction and stabilization of the transcripts. pM1h-IL3-Δ5'UTR, lacking the entire 27 nucleotides from the 5' UTR, was generated from pM1h-IL3-wt using a Quik-Change™ site-directed mutagenesis kit supplied by Stratagene. The reporter constructs pMxh-β-globin and pMxh-β-globin-ARE were provided by Dr H.H.Hirsch. The constructs contain a *PvuII* fragment of rabbit β-globin under the control of the Moloney murine leukaemia virus LTR. The 3' UTR of the former construct is from β-globin, and is replaced by the ARE-containing 3' UTR of IL-3 mRNA in the latter construct. All these constructs include the hygromycin B phosphotransferase (hph) cDNA under control of SV40 regulatory regions, as selection marker. Construct SRα3-MEKKΔ (Minden *et al.*, 1994), SRα3-MEK6DD (Stein *et al.*, 1996), pGEX-2T-*c-jun* (1–79) (Hibi *et al.*, 1993) and pGEX-2T-ATF2 (1–254) (Gupta *et al.*, 1995) were generously provided by Dr M.Karin, pCMV-JNK-APF (Gupta *et al.*, 1995) and pCMV-p38-AGF were provided by Dr R.J.Davis.

Northern blot analysis

Approximately 2.5×10^5 cells/ml in 15 ml of complete IMDM supplemented with 10% FCS and IL-3 were cultured overnight before the various experimental treatments. Total cytoplasmic RNA was isolated using the method of Gough (1988). A quantity of 20 μg total RNA was used for analysis, and hybridizations were performed as described previously (Nair *et al.*, 1992). ³²P-labelled antisense RNA probes were synthesized using a SP6 transcription kit (Boehringer Mannheim). The following riboprobes were used: pSP65-multi-CSF containing a 368-bp *HindIII*–*XbaI* IL-3 cDNA fragment; pGEM-actin containing a 567-bp *PstI* fragment of chicken β-actin cDNA as described (Nair *et al.*, 1994). pGEM-globin containing a 380-bp *HindIII*–*BamHI* fragment of rabbit β-globin cDNA. Quantification of the IL-3 and β-actin mRNA hybridization signals was performed with a PhosphorImager (Molecular Dynamics) using ImageQuant software as described previously (Banholzer *et al.*, 1997). The IL-3 signals were normalized to the respective β-actin reference signal after subtraction of the filter background.

RNase A/T1 protection assay

RNase A/T1 protection assays were performed as described previously (Stoecklin *et al.*, 1994). A quantity of 10 μg total cytoplasmic RNA was hybridized with a non-limiting amount of ³²P-labelled IL-3 and hygromycin-antisense RNA probes at 45°C for at least 18 h. Non-hybridized RNA was digested with RNase A (Boehringer Mannheim, 40 μg/ml) and RNase T1 (Boehringer Mannheim, 1000 U/ml) at 22°C for 30 min. After treatment with proteinase K, phenol/chloroform extraction and ethanol precipitation, the RNA was analysed by electrophoresis in a 6% polyacrylamide, 7 M urea gel. ³²P-labelled antisense RNA probes were synthesized using a SP6 transcription kit (Boehringer Mannheim), and full-length transcripts were purified by elution from a polyacrylamide gel. The IL-3 probe was as described for Northern blot analysis. Transcription template for hph was a 96-bp *EcoRI*–*PstI* fragment in pGEM-hygro (Nair *et al.*, 1994).

Nuclear run-on analysis

Nuclear preparation, transcription reaction and isolation of labelled RNA were carried out as described previously (Nair *et al.*, 1994). Aliquots of 1 μg of the following DNA probes were denatured and slot-blotted onto reinforced nitrocellulose (Schleicher & Schuell): (i) pGEM; (ii) pGEM-IL3, as described for Northern blot analysis; (iii) pGEM-actin, as described for Northern blot analysis; (iv) pGEM-GM-CSF, containing the 750-bp *BamHI*–*EcoRI* fragment of GM-CSF cDNA. The filters were hybridized with labelled nuclear RNA at 50°C for 24 h in the same solution described for Northern blot analysis, washed in the same way, and exposed on a PhosphorImager screen (Molecular Dynamics).

Immunoblot

Approximately 2.5×10^5 cells/ml in 15 ml of complete IMDM supplemented with 10% FCS and IL-3 were cultured overnight, and treated as indicated in the figures. Cell extracts were prepared by lysing 2.5×10^6 cells in 100 μl SDS sample buffer (2% SDS, 10% glycerol, 80 mM Tris pH 6.8, 0.15 M 2-mercaptoethanol, 0.02% bromophenol blue). The 30 μl extracts were subjected to SDS-PAGE, electrophoretically transferred to an Immobilon P membrane (Millipore), and the resultant membrane was incubated overnight with the appropriate primary antibody at 4°C, with gentle agitation after blocking with 5% skimmed milk. The protein was decorated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody and developed using the ECL system (Amersham). For further incubation with another primary antibody, the blot was stripped at 50°C for 30 min in a stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl pH 6.7.

Protein determination and in vitro kinase assay

The protein concentration of cell extracts was determined using a Bio-Rad D/C protein assay kit with bovine serum albumin (BSA) as the standard. For kinase assays, lysates containing 100 μg protein were used. The solid-state JNK assay was performed by incubating cell extracts with GST-*c-Jun* (1–79)–GSH agarose beads as described previously (Hibi *et al.*, 1993). For p38 MAPK assay, 100 μg cell lysate was immunoprecipitated with 3 μl of a specific anti-p38 MAPK antibody. Immunoprecipitates were then assayed for the kinase activity using GST-ATF2 (1–254) fusion protein as a substrate.

Acknowledgements

The authors would like to thank Dr M.Karin, Dr R.J.Davis and Dr H.H.Hirsch for the gifts of various plasmids and reagents. We also thank Drs A.Wyss and L.Brennan for their comments on the manuscript, Dr M.Karin for discussion and M.Colombi for his valuable help in preparing the figures. This work was supported by grant 31-40816.97 of the Schweizerische Nationalfonds to C.Moroni.

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*Received July 7, 1998; revised August 31, 1998;
accepted September 1, 1998*