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p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient

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The ERK, JNK/SAPK and p38/RK MAP kinase subtypes (reviewed in [1]) are differentially activated in mammalian cells by various stimuli, which elicit induction of immediate–early (IE) genes, such as c-*fos* and c-*jun* (reviewed in [1–3]), as well as phosphorylation of histone H3 [4] and HMG-14 [5]. Anisomycin and UV radiation have been suggested to induce c-*fos* and c-*jun* transcription *via* JNK/SAPK-mediated phosphorylation of TCF (ternary complex factor), for c-*fos* induction [6–8], and c-Jun and/or ATF-2 for c-*jun* induction [9–13]. We report here that anisomycin and ultraviolet radiation (UV) activate MAP kinase kinase-6 (MKK6) [14,15], p38/RK [16–18] and MAPKAP kinase-2 (MAPKAP K-2) [17–19]. By using the p38/RK inhibitor SB 203580 [20,21], we show that activation of p38/RK and/or its downstream effectors are essential for anisomycin- and UV-stimulated c-*fos*/c-*jun* induction and histone H3/HMG-14 phosphorylation, whereas JNK/SAPK activation and phosphorylation of c-Jun and ATF-2 are insufficient for these responses.

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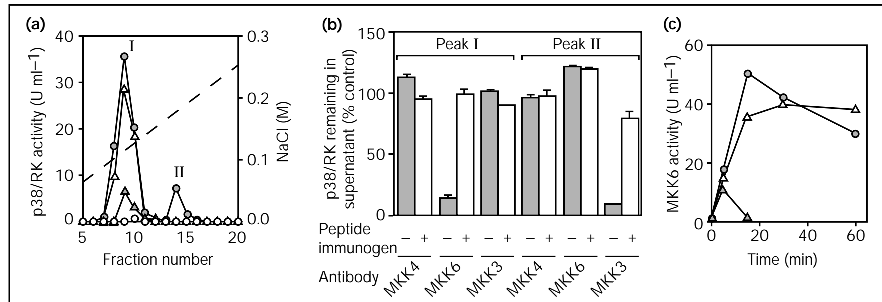
Results and discussion

The activation of kinase cascades in C3H 10T½ murine fibroblasts by various stimuli has been described previously [19,22,23]. We recently showed that anisomycin and UV radiation strongly activate MAPKAP K-2 in C3H 10T½ cells [19], implying that these stimuli must also activate the upstream kinase p38/RK (reviewed in [1]). To analyze this process in greater detail, lysates from cells stimulated with EGF (epidermal growth factor), anisomycin or UV radiation were subjected to Mono S chromatography, and fractions assayed for upstream activators of p38/RK. In parallel, these lysates were assayed for p38/RK and MAPKAP K-2 activities (data not shown), which correlated closely with the activity of the upstream activator of p38/RK (Fig. 1a). The major activator of p38/RK (peak 1) coeluted with MKK6 (Fig. 1a; [14,15]). This activity was immunoprecipitated specifically by anti-MKK6 antibodies but not by anti-MKK3 antibodies (Fig. 1b). The minor activator of p38/RK (peak II), seen only in response to anisomycin, was immunoprecipitated by anti-MKK3 antibodies but not by anti-MKK6 antibodies (Fig. 1b). Neither the major, nor the minor, p38/RK activator was immunoprecipitated by anti-MKK4 antibodies (Fig. 1b). MKK6 is also the major p38/RK activator in KB cells and THP-1 monocytes but not in PC12 cells [15]. An important role for MKK6 in activating p38/RK is evident from the fact that, although MKK3 does activate co-transfected over-expressed p38/RK, it only activates the endogenous enzyme very weakly, whereas transfected MKK6 activates endogenous p38/RK efficiently [14].

Time-course analyses showed that EGF activated MKK6 (Fig. 1c), p38/RK (data not shown) and MAPKAP K-2 (data not shown; see [19,23]) weakly and very transiently; activity returned to basal levels within 15 minutes. In contrast, anisomycin and UV radiation produced strong activation at all three levels of this cascade, and activity remained high at 60 minutes (Fig. 1c, and data not shown). Similar assays showed that okadaic acid activated this cascade more gradually, but to high levels, whereas TPA (12-O-tetradecanoylphorbol 13-acetate) did not activate this pathway at all (data not shown). These observations are exactly as expected from our previous analyses of two kinases, p45 and p55 [23], recently identified as MAPKAP K-2 [19].

The pyridinyl imidazole compound SB 203580 binds specifically to p38/RK and inhibits its activity, thereby preventing activation of its downstream kinase MAPKAP K-2 [20,21]. We found that SB 203580 inhibited p38/RK isolated from C3H 10T½ cells by Mono Q chromatography with an *in vitro* IC_{50} value (0.6 μM) identical to that required to inhibit p38/RK from other mammalian cells (data not shown). Consistent with this finding, SB 203580 strongly inhibited MAPKAP K-2 activation in C3H 10T½ cells in response to anisomycin (90 % inhibition) or UV radiation (80 % inhibition; data not shown). In contrast, activation of its upstream activator MKK6 was not inhibited and, if anything, was slightly augmented (data not shown); this may reflect ablation of negative-feedback influences normally originating from downstream kinases. To confirm its specificity, we also showed that SB 203580

Figure 1

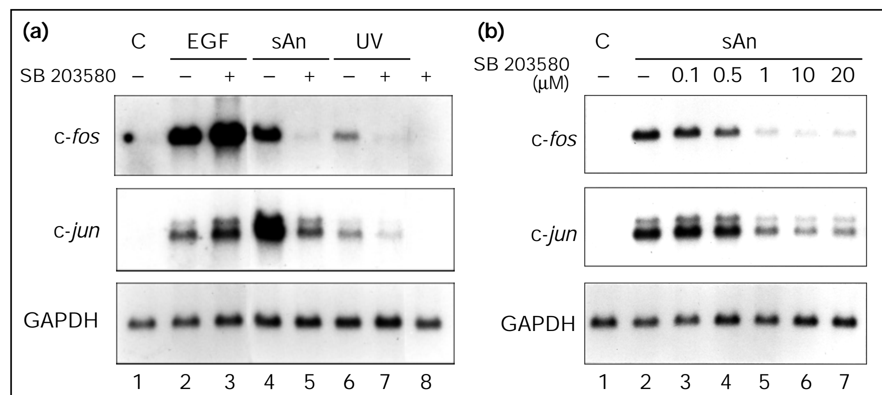


Activation of p38/RK activators by anisomycin, UV radiation and EGF in C3H 10T½ cells. **(a)** Mono S chromatography of p38/RK activators from C3H 10T½ cell extracts. Quiescent cells (open circles) or cells stimulated with anisomycin (50 ng ml^{-1}, 30 min; closed circles), UV radiation (400 J m^{-2}, 30 min; open triangles) or EGF (50 ng ml^{-1}, 5 min; closed triangles) were lysed, and aliquots (0.3 mg protein) chromatographed in a Pharmacia SMART system using 5 × 0.16 cm Mono S columns, as described [15,17,25]. The p38/RK activator kinase assay was identical to that described previously [25]. One unit of activity was the amount that increased the activity of MalE–XMpk2 by one unit per minute [26]. Subinhibitory concentrations of anisomycin (below those required to inhibit protein synthesis [27]) were used here to eliminate considerations of translational arrest; inhibitory concentrations also produced very strong activation of this cascade. **(b)** The major p38/RK activator seen in (a) was immunoprecipitated by anti-MKK6 antibodies. Aliquots of column fractions from peaks I and II in (a) were immunoprecipitated, as described previously, with anti-MKK4 [26], anti-MKK6 [15], or anti-MKK3 antibodies [15]. The graph shows the p38/RK activity remaining in the supernatant after immunoprecipitation in the presence (+) or absence (–) of the appropriate competing peptide immunogen, relative to control incubations (100 %) where antibody was omitted. The p38/RK activator activity was assayed as described above. **(c)** Time-course analysis of MKK6 activation by anisomycin, UV radiation and EGF. Quiescent cells were stimulated for the times indicated with anisomycin (50 ng ml^{-1}; closed circles), UV radiation (400 J m^{-2}; open triangles) or EGF (50 ng ml^{-1}; closed triangles), and lysates were subjected to Mono S chromatography; MKK6 activity was quantitated as described above.

did not affect ERK activation in response to EGF or TPA (data not shown), or JNK/SAPK activation in response to EGF, anisomycin or UV radiation (data not shown; see also Fig. 3a).

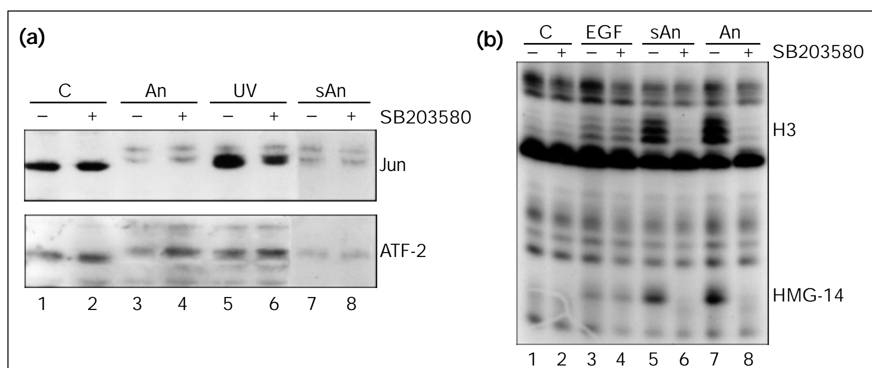
The time-course and relative strengths of induction of c-*fos* and c-*jun* in response to EGF, anisomycin and UV radiation in C3H 10T½ cells have been described [22,23]. SB 203580 strongly inhibited the induction of c-*fos* and c-*jun* by anisomycin and UV radiation (Fig. 2a). The extent of inhibition for both genes (80–90 %) was similar to the inhibition of p38/RK under these conditions (see above). By reprobing these blots, we also found that SB 203580 blocked anisomycin- and UV-stimulated induction of *jun*B by 80–90 %, but was less effective (~65 % inhibition) at blocking *jun*D induction (C.A.H. and L.C.M, unpublished observations). However, SB 203580 did not inhibit the induction of c-*fos* and c-*jun* in response to EGF (Fig. 2a); in fact, we consistently observed augmented c-*fos* and c-*jun* mRNA levels under these conditions. This enhancement was not attributable to increased stability of these transcripts (data not shown), but could conceivably have resulted from increased activation of other MKK6-triggered routes to these genes (see above). Similarly, the

Figure 2



Effect of SB 203580 on the induction of c-*fos* and c-*jun* by EGF, anisomycin and UV radiation. **(a)** Northern blot analysis of c-*fos* and c-*jun* expression. C3H 10T½ cells incubated in the presence (+) or absence (–) of 20 μM SB 203580 for 15 min, were then stimulated with EGF (50 ng ml^{-1} for 30 min); anisomycin (sAn; 25 ng ml^{-1} for 45 min); UV radiation (UV; 200 J m^{-2} for 45 min), as indicated. Lane 1, control (C; unstimulated); lane 8, SB 203580 (20 μM, 60 min). The optimal times for each stimulus, and the relative strength of induction of c-*fos* and c-*jun* at these time points were determined by time-course analysis over 60 min, as described [22]. Procedures for C3H 10T½ cell culture, RNA extraction, northern blotting and the probes used have been described ([22] and references therein). **(b)** Dose-dependent inhibition of anisomycin stimulated induction of c-*fos* and c-*jun* by SB 203580. Lane 1, control (unstimulated); lanes 2–7, cells stimulated with anisomycin (sAn; 25 ng ml^{-1}) for 45 min. SB 203580 at the indicated concentrations was added 15 min before stimulation with anisomycin.

Figure 3



Effect of SB 203580 on the phosphorylation of nuclear proteins. **(a)** SB 203580 has no effect on anisomycin- or UV-stimulated phosphorylation of c-Jun or ATF-2. C3H 10T½ cells were incubated in the presence (+) or absence (–) of SB 203580, and either unstimulated (lanes 1 and 2) or stimulated with anisomycin at inhibitory (An; lanes 3 and 4) or subinhibitory (sAn; lanes 7 and 8) concentrations, or with UV radiation (lanes 5 and 6), as indicated. The cells were then lysed and nuclear extracts [28] were run on SDS–polyacrylamide gels, and western blotting was carried out using anti-c-Jun or anti-ATF-2 antibodies, as indicated. The anti-c-Jun antibody (Affiniti) was used at 1:1000 dilution, and the anti-ATF-2 antibody (Santa-Cruz) was used at 1:500 dilution. After washing [22], blots were incubated with sheep second antibody coupled to horseradish peroxidase (1:10,000 in TBST) for 1 h, washed as before and visualized using the Renaissance detection system (NEN). Migration of phosphorylated c-Jun and ATF-2 is retarded on these gels. **(b)** SB 203580 inhibits anisomycin-stimulated but not EGF-stimulated histone H3 and HMG-14 phosphorylation. Confluent quiescent C3H 10T½ cells were labelled with ^{32}P-phosphate as described [4,5], incubated in the presence (+) or absence (–) of 20 μM SB 203580 for 15 min, and then stimulated with EGF (50 ng ml^{-1}), subinhibitory anisomycin (sAn; 50 ng ml^{-1}) or inhibitory anisomycin (An; 10 mg ml^{-1}), as indicated. Lanes 1 and 2: control (C; unstimulated) cells. After sequential extraction, proteins from the chromatin-enriched fraction were extracted, and resolved on acid–urea gels [4,5] in which histone H3 resolves into multiple forms according to its state of modification, principally acetylation [5].

induction of c-*fos* and c-*jun* by TPA was unaffected or slightly augmented in the presence of this inhibitor (data not shown). Dose-dependence studies showed that SB 203580 effectively ablated c-*fos* and c-*jun* induction at low micromolar concentrations (Fig. 2b) similar to those required to inhibit p38/RK and its activation of MAPKAP K-2. Thus, induction of c-*fos* and c-*jun* by anisomycin and UV radiation, but not by EGF or TPA, was substantially inhibited under conditions where the p38/RK MAP kinase cascade was inhibited by SB 203580.

We then asked if the phosphorylation of transcription factors that control IE genes was affected by the inhibitor. For these studies, we focused on the transcription factors c-Jun and ATF-2, which are differentially phosphorylatable by JNK/SAPKs and p38/RK, and implicated in c-*jun* induction [9–12]. Our results showed conclusively that anisomycin- and UV-stimulated c-Jun and ATF-2 phosphorylation were unaffected by SB 203580 (Fig. 3a), an observation which agrees with the finding that under these conditions JNK/SAPKs remain strongly active. Treatment with anisomycin elicited stoichiometric and prolonged c-Jun phosphorylation (data not shown), and some loss of immunoreactivity, possibly resulting from degradation (Fig. 3a). These experiments were repeated in human MRC-5 cells with identical results (data not shown). Furthermore, we found, as expected, that EGF-stimulated phosphorylation of c-Jun and ATF-2, which is much weaker and more transient, was unaffected by SB 203580 (data not shown). Note that in this study we have avoided using transfected epitope-tagged or over-expressed proteins, which have the potential to produce misleading results.

Finally, we investigated the effects of SB 203580 on the nucleosomal response, the phosphorylation of histone H3 [4] and HMG-14 [5], which is thus far the only nuclear event that we have found to be invariably associated with IE gene induction [22]. As shown in Figure 3b, the effect of SB 203580 on the nucleosomal response closely paralleled its effects on IE gene induction: SB 203580 did not affect histone H3 or HMG-14 phosphorylation elicited by EGF, but completely ablated phosphorylation produced by either subinhibitory or inhibitory concentrations of anisomycin. Thus, in anisomycin-stimulated SB 203580-treated cells, JNK/SAPKs remained active and c-Jun and ATF-2 remained highly phosphorylated (Fig. 3a), whereas c-*fos* and c-*jun* induction (Fig. 2a,b) and the nucleosomal events (Fig. 3b) were strongly inhibited, emphasizing further the tight correlation between IE gene induction and the nucleosomal response.

These results, in accord with recent and emerging evidence [20,21,24], confirm the high specificity of SB 203580 as an inhibitor of p38/RK and prove that it does not interfere non-specifically with IE gene induction, the phosphorylation of transcription factors c-Jun and ATF-2, or with the nucleosomal response. Furthermore, they show that p38/RK is clearly essential for nuclear responses induced by anisomycin and UV radiation, but not by EGF. An important corollary here is that, although ATF-2 is a substrate for p38/RK *in vitro*, its phosphorylation in these experiments cannot be attributed to p38/RK. Under conditions where ERKs are inactive, JNK/SAPKs have been proposed to mediate c-*fos* activation by phosphorylating TCFs [6–8], although this has not been verified in intact

untransfected cells. Our data suggest that, even if this occurs *in vivo*, it is insufficient for c-*fos* activation; events mediated by p38/RK or its downstream kinases are also required. The recent observations that selective activation of p38/RK induces a reporter construct driven by the c-*fos* serum-response element, and that p38/RK phosphorylates Elk-1 *in vitro* [14], may explain the p38/RK-dependence of c-*fos* induction seen here. It is unclear whether the stress-induced nuclear events require p38/RK itself or its downstream kinases. p38/RK is clearly capable of phosphorylating transcription factors, such as CHOP, in intact cells — an event blocked by SB 203580 [24]. Alternatively, or in addition, the nuclear response may require not p38/RK but its downstream effector MAPKAP K-2. For instance, MAPKAP K-2 mediates phosphorylation and activation of CREB (cyclic AMP response element-binding protein) in response to cytokines and cellular stress ([25]; J. Rouse and P.C., unpublished observations), an event which may participate in activating c-*fos* through upstream CRE sites (reviewed in [2]).

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