Characterisation of signalling pathways leading to the serum response element

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The Serum Response Element (SRE) is required for activation of *c-fos* transcription in response to diverse extracellular signals. At a *c-fos* promoter, SRE is bound by a transcription factor complex composed of SRF (serum response factor) and TCF (ternary complex factor) proteins. Transcriptional activation by SRF and TCF is responsive to distinct signalling pathways. TCF activity is regulated by phosphorylation by MAP kinases, while SRF activity is potentiated by RhoA-dependent signalling pathways. Transiently transfected SRF reporter gene templates are strongly activated by foetal bovine serum and agents such as lysophosphatidic acids (LPA) acting through heterotrimeric G protein-coupled receptors. Immunofluorescence and RNAse protection assays were used to investigate the regulation of the SRF-controlled reporter gene microinjected or stably transfected into NIH3T3 cells. Unlike transiently transfection assays, these assays revealed reporter activation by receptor tyrosine kinases and phorbol esters. However, in contrast to signals induced by the latter agents, inhibitor studies have demonstrated that polypeptide growth factor- and TPA-induced SRF activation requires phosphatidylinositol 3-kinase activity.

Key words: transcription, SRE , SRF, *c-fos,* gene expression, RhoA

INTRODUCTION

Treatment of quiescent cells in culture with serum mitogens or growth factors allows cells to enter cell cycle and subsequently go through a round of DNA synthesis and cell division [1]. All early events involved in this process depend on the transcriptional activation of a subset of so-called immediate-early genes (for review see [2, 3]). The response of the cell to a particular stimulus is dependent on many different genes. Therefore, understanding the mechanisms of gene activation is of great importance. The main focus of this study is the transcription factor SRF (serum response factor) and the signalling pathway that controls its activity [4, 5]. The prototype of the SRF target gene is the *c-fos* proto-oncogene. At a *c-fos* promoter, SRF binds as a part of a complex with another transcription factor, TCF (ternary complex factor) [6]. The activity of both SRF and TCF is regulated by the small GTPase RhoA and MAP kinases, respectively [7, 8]. In this particular study, an SRF-controlled reporter gene has been used in order to dissect the signalling pathways that activate SRF, but not TCF. Some reports suggest that chromatin modifications such as acetylation and phosphorylation are actively involved in the activation of transcription [9, 10]. Attempts have been made to establish whether a stably integrated reporter would behave in a similar manner compared to the transiently transfected one. The study has revealed that in NIH3T3 cell line the stably integrated SRF-controlled reporter gene is activated by receptor tyrosine kinases and phorbol esters, in addition to serum and LPA as previously observed in transfection assays. It is proposed that this uncharacterised signalling pathway to SRF is dependent on functional PI-3 kinase.

MATERIALS AND METHODS

Cells lines, transfections and stimuli

SREFosHA cells are a NIH3T3 line with an integrated 3D.AFosHA reporter [9]. NIH3T3 cells in 6 cm dishes were transiently transfected using LipofectAMINE (Life Technologies Inc.) according manufacturer's recommendations. 0.3 μg 3D.AFos reporter and 1.7 μg MLV128β carrier plasmid were used per dish; cells were starved in 0.5% FBS for 36 h and then stimulated with FBS (Gibco-BRL), LPA, TPA (Sigma), or PDGF (Calbiochem).

RNase protection assay

RNA preparation and RNAse protection assays were as described in [7]. GAPDH and 3D.AFos probes were as described [11].

Immunoblotting and immunofluorescence

Total cell lysates were prepared as follows: cells were rinsed twice with ice-cold PBS and lysed into a 2x Laemmli sample buffer. Equal amounts of lysate were resolved by SDS-PAGE transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore) and probed with anti-HA antibodies (Boehringer Mannheim). Horseradish peroxidase conjugated anti-mouse goat antibodies were from DAKO; ECL detection reagents were from Amersham Pharmacia Biotech. For immunofluorescence, cells were grown on glass coverslips, serum-starved and then stimulated with various agents. The cells were fixed with 4% formaldehyde in PBS for 15 min and then permeabilized for 10 min in 0.3% Triton X-100 in PBS. Antibodies were diluted 1:100 in PBS, 0.5% NP-40, and 5% FCS; cells were washed 4 times in PBS before mounting. The antibodies used were as follows: anti-HA Y-11 (Santa Cruz) and anti-rabbit Texas Red (Molecular Probes).

RESULTS AND DISCUSSION

Activation of the stably integrated SRF reporter gene

It has been previously reported that the transiently transfected SRF-dependent reporter can be activated by serum and LPA and is refractory to receptor tyrosine kinases and phorbol esters [7]. It has also been shown that in transfection assays the small GTPase, RhoA, is necessary and sufficient to activate SRF-controlled reporter genes. However, it remained unclear whether a chromosomally integrated reporter would behave in a similar manner compared to the transfected reporter. A NIH3T3-derived cell line carrying a stably integrated SRF-controlled reporter gene, 3D.AFos, was used. The 3D.AFos reporter comprises the human c-fos transcription unit controlled by a chimeric promoter containing a cytoskeletal actin TATA region (minimal promoter) and three upstream SRF binding sites [12]. RNase protection assay was used, since it allows monitoring both the kinetics and magnitude of induction of mRNAs of interest. To extend the data obtained by transfection experiments [7], other stimuli such as the polypeptide factor PDGF-BB and the phorbol ester TPA were used, in addition to serum and LPA. In contrast to serum and LPA which induced activation of both integrated and transiently transfected reporters, PDGF and TPA strongly activated the integrated reporters (see Fig. 1A). The peak of RNA accumulation induced from an integrated reporter by serum LPA and TPA occurred at 60 min, contrasting to PDGF induction which peaked around 30 min and declined rapidly thereafter. The magnitude of induction at 30 min was comparable for all stimuli used. To make sure that the observed differences were not single clone artefacts arising from integration positional effects, two additional clones were examined. PDGF- and TPA-induced SRF reporter activity was detected in both clones (data not shown).

To confirm the somewhat unexpected behaviour of integrated reporter clones measured by RNase protection analysis, it was tested whether the integ-

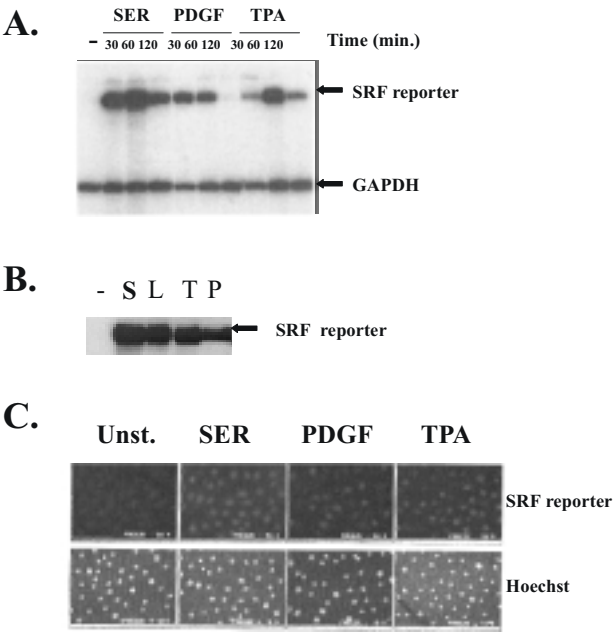

Fig. 1. Activation of the stably integrated SRF reporter gene by different stimuli. A. Induction of SRF reporter at RNA level. Serum-deprived SRE.FosHA cells were stimulated with 15% Foetal Calf Serum (SER), 25 ng/ml PDGF or 50 ng/ml TPA for times indicated in the figure. Transcripts of the reporter and control gene, GAPDH, were analysed by RNAse protection; nuclease-resistant fragments derived from the two RNAs are indicated in the figure. **B. Induction of SRF reporter at protein level.** Serum-deprived SRE.FosHA cells were stimulated for 60 min with 15% Foetal Calf Serum (S), 10 μM LPA (L), 25 ng/ml PDGF (P) or 50 ng/ml TPA (T). Whole cell lysates were fractionated by SDS-PAGE, transferred to PVDF membrane before immunoblotting for SRF reporter protein with anti-HA antibody. **C. Analysis of SRF reporter activation by immunofluorescence**. SRE.FosHA cells were seeded on cover-slips, starved for 24 h in 0.5% FBS and then stimulated for 60 min with Foetal Calf Serum (SER), 25 ng/ml PDGF or 50 ng/ml TPA prior to fixation and staining for expression of the SRF reporter with anti-HA antibody. Cell nuclei were stained with Hoechst

rated reporter is expressed at the protein level. Several single clones were analysed by western blotting using the anti-HA-tag antibody. Cells were starved and then stimulated with serum, LPA, PDGF or TPA and tested for the expression of the HA-tagged protein (see Fig. 1B). Protein accumulation was readily detectable 60 min following stimulation. As in the case of RNA, the levels of serum-, LPA- and TPA-induced SRF reporter activity were comparable, although in PDGF-treated cells the induction of the SRF reporter protein was lower. These data demonstrate, however, that the integrated SRF reporter induction can be detected both at protein and RNA level following serum, LPA, PDGF or TPA stimulation.

Analysis of the SRF reporter activation by immunofluorescence

To test whether reporter activity was uniform throughout the cell population, a single cell based technique employing immunofluorescence was used. This technique allows comparison of the activity of the reporter gene between single cells as well as the whole population of cells. However, a disadvantage of this technique is that it allows only an approximation of the levels of expressed reporter. Cells were seeded on glass coverslips, starved for 24 h in 0.5% FBS and then stimulated with serum, LPA, PDGF and TPA for 60 min, fixed and tested for the presence of the SRF reporter expression using anti-HA and fluorescence conjugated antibodies. As is shown in Fig. 1C, serum induced SRF reporter expression in almost all cells and the level of activity was relatively homogeneous within the cell population. A similar pattern was observed after LPA stimulation, although the signal was much weaker, consistent with that observed by western blotting (compare Fig. 1B and 1C). A different distribution of the signal was observed after PDGF and TPA stimulation: approximately half of the cells exhibited high reporter activity, yet some cells showed very weak immunofluorescence. The lower mRNA and protein measurements observed previously in PDGF- and TPA-treated cells (see above) could possibly be explained by the fact that only a fraction of the cells respond to the stimulation. These data suggest that results obtained using total cell lysates or total RNA represent an average signal from the cell population.

Induction of the transfected or microinjected (extrachromosomal) reporter

The inducibility of transfected SRF reporter was examined using lipofectamine transfection technique. Following the transfection of SRF reporter, NIH3T3 cells were starved and then stimulated with various

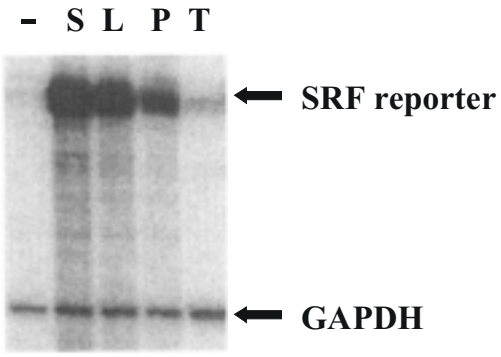

Fig. 2. Activation of transiently transfected SRF reporter gene. NIH3T3 were were transfected with the SRF reporter gene 3D.AFos template. Cells were maintained in 0.5% FBS for 24 h and then stimulated for 30 min with Foetal Calf Serum (SER), 10 μM LPA (L), 25 ng/ml PDGF (P) or 50 ng/ml TPA (T). Transcripts of the SRF reporter and control gene, GAPDH, were analysed by RNAse protection

agents. As is shown in Fig. 2, the SRF reporter was activated at a high level by serum and LPA, a barely detectable level by PDGF, while no detectable level of activation was observed by TPA. This data show that, in contrast to serum and LPA, TPA does not result in the activation of the transfected SRF reporter in any conditions tested. However, PDGF treatment resulted in low but reproducible induction of the reporter.

Another way to deliver the reporter into the cell is to inject directly the DNA template into the cell nucleus. This technique allows the monitoring of reporter activity within hours of injection, in contrast to traditional transfection methods with which reporter activity is measured one or two days after transfection. Of course, microinjection is a substantially different method of DNA delivery to the cell, therefore it was questioned whether this would result in different activation characteristics for the reporter when compared to conventional lipofectamine transfection techniques. As expected, serum addition caused induction of the SRF reporter, however, in contrast to some transfection experiments, PDGF treatment also resulted in a significant reporter expression (data not shown). It is worth noting, however, that only 60–70% of the cells responded to PDGF, and the relative reporter level was still lower than that achieved with serum. Taken together, these data suggest that SRF-controlled reporter activation by PDGF registers only in more sensitive assays such as microinjection or RNase protection.

PI-3 kinase activity is required for PDGF-, but not for serum- or LPA-induced SRF activation

PI-3 kinase has been implicated in SRF activation in transfection experiments in HeLa cells [13].

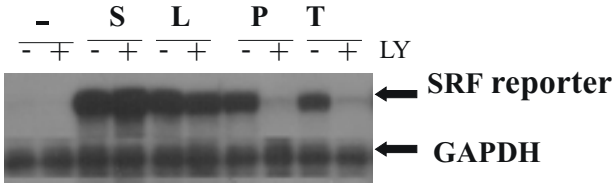

Fig. 3. SRF-linked signalling pathways dependence on PI-3 kinase. Serum-deprived SRE.FosHA cells were pretreated with 20 μM LY294002 (+) and Foetal Calf Serum (S), 10 μM LPA (L), 25 ng/ml PDGF (P) or 50 ng/ml TPA (T). Transcripts of the SRF reporter and control gene, GAPDH, were analysed by RNAse protection.

Therefore, it was tested whether activation of the SRF reporter was dependent on PI-3 K activity. For this reason, cells were pre-treated with 20 μM LY 294002 and stimulated for 30 min with serum, LPA, PDGF or TPA. The activity of the stably integrated SRF-controlled reporter was determined using RNase protection assay (Fig. 3). Both the PDGF- and TPA-induced reporter activity was substantially inhibited by pre-treatment of PI-3 K inhibitor LY294002, however, serum- and LPA-induced activity was only insignificantly affected by this inhibitor. These data suggest that SRF can be activated by two distinct signalling pathways, one of which requires PI-3 K activity. To confirm these findings, another structurally different inhibitor of PI-3 K, Wortmannin, was used. Wortmannin pre-treatment results in the same pattern of inhibition of the SRF reporter gene as was observed with LY294002 (data not shown). However, LPA-induced SRF activity was slightly more sensitive to Wortmannin than to LY294002. Taken together, these data show that PI-3 K activity is dispensable for serum- and LPA-, but, in contrast, is required for PDGF- and TPA-induced SRF reporter gene activation. The requirement of PI-3 kinase for PDGF-induced SRF reporter gene activity is not totally surprising, since PDGF strongly activates PI-3 K. It has been suggested that the activity of the guanine nucleotide binding protein Vav, an exchange factor for Rac, is directly controlled by PI-3 K products [14]. As Rac itself is sufficient to activate SRF, it is tempting to speculate that PDGF signalling to SRF is mediated through Rac. TPA-induced signals to SRF remain elusive, since TPA does not activate PI-3 kinase. Further experiments are required to resolve these issues.

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SERUMO ATSAKO ELEMENTĄ (SRE) AKTYVUOJANTYS SIGNALINIAI KELIAI

S a n t a u k a

Skirtingų išorinių signalų indukuoto *c-fos* aktyvavimui yra būtinas SRE. *c-fos* promotoriuje SRE susijęs su transkripcijos veiksnių kompleksu, susidedančiu iš SRF ir TCF. SRF ir TCF yra aktyvuojami skirtingų signalinių kelių: TCF reguliuojamas MAP kinazių, o SRF aktyvumas – nuo Rho priklausomu signaliniu keliu. Laikinai transfekuotas SRF genas-reporteris aktyvuojamas veršelių serumu ar kitais agentais (pvz., LPA), veikiančiais per G baltymų receptorius. Šiame darbe buvo tiriamas transfekuotas ar injekuotas SRF geno-reporterio aktyvumas imunofluorescencijos bei RNR apsaugos būdu. Paaiškėjo, kad SRF geno-reporterio indukavimas augimo veiksniais ir forbolio esteriais priklauso nuo fosfatidilinozitol 3-kinazės aktyvumo.