v-Fps-responsiveness in the Egr-1 promoter is mediated by serum response elements

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ABSTRACT

Egr-1, a mitogen-responsive transcription factor, is rapidly induced by v-Fps in the absence of protein synthesis. Thus, Egr-1 is a primary response to the protein-tyrosine kinase activity of v-Fps. To determine the v-Fps-responsive elements in the Egr-1 promoter, deletion mutants of the Egr-1 promoter were used in transient expression assays. A v-Fps expression vector was cotransfected into NIH 3T3 cells with chloramphenicol acetyl transferase (CAT) gene expression vectors under the control of the Egr-1 promoter or the Egr-1 promoter containing various deletions. Responsiveness to v-Fps was restricted to a region that contained repeated CC(A/T)₆GG sequences, known as CARG boxes. CARG boxes form the core of serum response element (SREs). v-Fps-induced Egr-1 promoter activation was lost by sequential removal of four tandemly repeated SREs. This region, containing four SREs, was found to be sufficient for maximal Egr-1 induction by v-Fps when placed upstream from a heterologous promoter. Individual SREs from this region were able to respond to v-Fps, however, the activation of the individual SREs was lower than that observed for the clustered SREs. These data suggest that v-Fps-responsiveness in the Egr-1 promoter is mediated by SREs.

INTRODUCTION

Fujinami Sarcoma Virus (FSV) encodes a phosphoprotein, v-Fps, with a kinase activity that phosphorylates proteins on tyrosine residues (1). The *v-fps* gene is a fusion of viral *gag* and cellular *fps* gene sequences (2). The resulting fusion *gag-fps* gene product has a constitutively active protein-tyrosine kinase activity relative to the cellular *fps* gene product (3). The resulting elevated protein-tyrosine kinase activity of v-Fps causes transformation both in

vivo and in vitro (2). In previous efforts designed to elucidate the pathways through which v-Fps transforms cells, we demonstrated that v-Fps induces expression of the 9E3/CEF4 cytokine gene via an intracellular signaling pathway that is dependent on protein kinase C (4) and a G-protein (5). To further characterize intracellular signaling by v-Fps we have looked at the expression of the mitogen responsive immediate early gene Egr-1 [also known as *zif/268* (6), *Krox 24* (7), *TIS 8* (8), CEF5 (9), NGFI-A (10) and 9II-6 (11)]. Egr-1 expression is activated in response to all mitogenic stimuli examined to date (for review, see 12). Egr-1 was recently shown to be induced by v-Fps (11, 13). The Egr-1 gene encodes a transcription factor (14, 15) that recognizes a specific DNA target sequence found upstream of several cellular genes (16, 17). The sequence recognized by Egr-1 is also recognized by the tumor suppressor Wilms' tumor gene product (18, 19). Thus, it is possible that Egr-1 controls secondary responses to v-Fps leading to the transformed phenotype. Data presented in this report characterize the mechanism through which v-Fps transcriptionally activates Egr-1.

MATERIALS AND METHODS

Cells and culture conditions

Normal rat 3Y1 cells, and 3Y1 cells transformed by a temperature sensitive derivative of Fujinami Sarcoma Virus NY225 (20, 21) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum. At the non-permissive temperature (40°C) the cells are non-transformed, where at the permissive temperature (35°C) the cells assume the transformed phenotype. Confluent cell cultures were made quiescent by maintaining the cultures in conditioned medium for four days. NIH 3T3 cells were obtained from the American Type Culture Collection and were also maintained in DMEM supplemented with 10% calf serum.

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Materials

Cycloheximide, phorbol dibutyrate and chloramphenicol were obtained from Sigma. (¹⁴C)-butyryl-co-enzyme A, was obtained from New England Nuclear.

RNA analysis

RNA for Northern Blot analysis was extracted as described by Hatch and Bonner (22) with modifications as described previously (23). Hybridizations were carried out using radiolabeled Egr-1 DNA and washings of the filters were performed as described by Davis et al. (24).

Plasmid constructions

pIV2.3 contains a *gag-fps* fusion gene cloned into SV2neo vector (25). The construction of pEgr-1 P1.2, pΔEgr-1 P1.2, pE425, pΔE425, pE395, pE359, pE342 and pE125 was previously described (26, 27, 28). pE425/250TKCAT was obtained by cloning a Hind III-SmaI fragment from pE425 spanning a region from -425 to -250 of the Egr-1 promoter, into the BamHI site of pTK35CAT. pTK35CAT contains the minimal promoter of the thymidine kinase gene of herpes simplex virus (29). pE395/250TKCAT, pE359/250TKCAT and pE342/250TKCAT were constructed as pE425/250TKCAT using a Hind III-SmaI fragment from pE395, pE359 and pE342 respectively. pTKCATSRE1-2 was obtained by cloning a Hind III-Hae II fragment from pE425/250TKCAT containing the first and second most distal SREs, into the Hind III-BamHI site of pTK35CAT. pTKCATSRE1,2,3 and 4, constructs contained double stranded oligonucleotides corresponding to the four most distal SREs, were synthesized on an Applied Biosystems oligonucleotide synthesizer. The oligonucleotides were designed to contain the core CArG box along with seven base pairs of the 5' and 3' flanking sequences of each SRE (30), along with 5'SalI and 3'BamHI restriction sites. The strands were annealed as described (26), and all SREs were cloned into the SalI-BamHI site of pTK35CAT.

Fig. 1. v-Fps induces increased levels of Egr-1 RNA independent of protein synthesis. Quiescent NY225-3Y1 cells were shifted from the non-permissive (40°C) to the permissive temperature (35°C) for v-Fps for 30 min in the absence or presence of cycloheximide (CHX) (10 μg/ml). RNA was harvested, electrophoresed and hybridized to radiolabeled Egr-1 DNA as described in Materials and methods. Egr-1 RNA levels were also examined in parental 3Y1 cells under the same conditions. Phorbol dibutyrate (PBt2) (0.1 μg/ml) was used to induce Egr-1 RNA (8) in 3Y1 cells as a positive control.

Cell transfections

NIH 3T3 cells were plated at 5×10^5 cells in 100mm dishes 20 hours prior to transfection. Transfections were performed using the CaPO₄ method as described previously (27). Transfection mixtures contained 30 μg of DNA, 5 μg of pIV2.3-fps or SV2-neo, 5 μg of the reporter CAT plasmid and 20 μg of carrier salmon sperm DNA. To insure equal transfection efficiency, all DNAs used for transfection were twice banded in cesium chloride gradients and all experiments reported here were performed from DNAs from a single DNA preparation.

CAT-assay

Transfected cultures were processed as described (27). CAT activity in cell extracts, using equal amounts of protein (31), was determined using the 'fluor diffusion assay' (32).

Fig. 2. Egr-1 promoter activation by v-Fps. pEgr-1 P1.2 contains the CAT gene under control of the Egr-1 promoter (23). CAT activity was assayed in extracts from NIH 3T3 cells that had been transfected with pEgr-1 P1.2 and either pIV2.3, a v-Fps expression vector (24), pSV2neo, the vector used to construct pIV2.3, or salmon sperm carrier DNA. Transfections were performed using the calcium phosphate co-precipitation technique as described in Materials and methods. 20 h later the cells were washed and CAT activity was assayed 24 h after washing using the fluor diffusion assay (37). a. Diagram of constructs used. Bent arrow represents translational start site. b. Relative CAT activity. Values were normalized to the CAT activity generated in the presence of pIV2.3 (v-Fps) which was assigned a value of 100. Data are the mean of at least four experiments.

RESULTS

The induction of Egr-1 expression is a primary response to the protein-tyrosine kinase activity of v-Fps

The induction of 'primary response' genes [those induced in the absence of protein synthesis (33)] is a useful early phenotype for the study of intracellular signal transduction pathways. A primary response to all mitogenic stimuli examined thus far is the induction of the transcription factor Egr-1 (12). We therefore examined

whether v-Fps-induced Egr-1 gene expression was sensitive to the protein synthesis inhibitor cycloheximide. To examine v-Fps-induced Egr-1 expression, we used 3Y1-NY225 cells which express a temperature-sensitive v-Fps mutant whose kinase activity is rapidly activated after temperature shift (20, 21). 3Y1-NY225 cells maintained at the non-permissive temperature for v-Fps (40°C) were shifted to the permissive temperature (35°C) and levels of Egr-1 RNA were determined using 'northern-blot' analysis. As demonstrated previously (11, 13), the induction of Egr-1 by v-Fps could be detected within 30 min (Figure 1). The induction of Egr-1 by v-Fps was not blocked by the protein synthesis inhibitor cycloheximide (Figure 1) and thus was independent of protein synthesis. The parental 3Y1 cells did not show increased levels of Egr-1 mRNA upon temperature shift (Figure 1). Thus, a primary response to increased v-Fps kinase activity is the induction of the mitogen-responsive transcription factor Egr-1.

Fig. 3. Deletion mutant analysis to the Egr-1 promoter reveals a v-Fps-responsive region. NIH3T3 cells were transfected with pEgr-1 P1.2 and derivatives containing various Egr-1 promoter deletions (23) along with pIV2.3. The location of *cis* transcriptional control elements in the Egr-1 promoter are shown in the diagram of the parental pEgr-1 P1.2 plasmid from which the deletion mutants were constructed. a. CAT expression vectors used. b. CAT activity was determined and normalized to the amount of CAT activity generated in the presence of pIV2.3 and pEgr-1 P1.2 (Egr1) as in Figure 2 and assigned a value of 100. Basal CAT activity generated by pEgr-1 P1.2 in the absence of v-Fps was equivalent to the CAT activity observed for pΔEgr-1 P1.2 (ΔEgr1). Data are the mean of at least four experiments.

Fig. 4. The four SREs contained between -425 and -250 are sufficient for conferring v-Fps-responsiveness. NIH 3T3 cells were transfected with pIV2.3 and constructs containing the deletions of pE425, pE395 and pE359 fused to the herpes thymidine kinase promoter contained in pTK35CAT (29), and CAT activity was assayed as in Figures 2 and 3. a. CAT expression vectors used. b. CAT activity was determined from cells transfected with pEgr-1 P1.2 deletion constructs and corresponding pTK35CAT constructs. Values were normalized to the amount of CAT activity generated in the presence of pIV2.3 and pEgr-1 P1.2. Solid and hatched bars represent pEgr-1 P1.2 and HSV-TKCAT deletion constructs respectively. Data are the mean of at least four independent experiments.

Activation of the Egr-1 promoter by v-Fps

To characterize the mechanism through which v-Fps activates Egr-1, we looked at the responsiveness of Egr-1 promoter sequences to v-Fps. pEgr-1 P1.2 contains Egr-1 promoter sequences between -957 and $+248$ relative to the transcriptional start site (26), cloned into pCAT3M (28) upstream from the CAT gene (Figure 2a). pEgr-1 P1.2 was cotransfected into NIH 3T3 cells with pIV2.3 (25), which expresses v-Fps under the control of the SV40 promoter (Figure 2a). 48 hours post-transfection, cell extracts were assayed for CAT activity. As shown in Figure 2b, expression of the CAT gene from pEgr-1 P1.2 was dramatically increased in the presence of v-Fps. Cotransfection of pEgr-1 P1.2 with the parental vector (pSV₂-neo) had no significant effect on CAT gene expression (Figure 2b). Thus, v-Fps is able to increase transcription from the Egr-1 promoter.

v-Fps-responsiveness resides in a region containing multiple SREs

To investigate the mechanism through which v-Fps activates the Egr-1 promoter, we used several constructs containing deletions in the Egr-1 promoter of pEgr-1 P1.2 (Figure 3a). pΔEgr-1 P1.2,

which lacks all the SREs with their core CarG boxes, but retains AP-1 and SP-1 sites (26, 27), was cotransfected with pIV2.3 (v-Fps). As shown in Figure 3b, deletion of this region containing 6 CarG boxes reduced CAT activity to basal levels. Deletion of the AP-1 and Sp-1 sites retained in pΔEgr P1.2 had no effect on CAT expression (see construct pE425, Figure 3a and 3b). Sequential deletion of the SREs from the 5' end of pE425 resulted in progressive loss of CAT activity. Deletion of two SREs immediately upstream of the Egr-1 TATA box, had no effect on the levels of activation. These data show that the Egr-1 upstream sequences contain a v-Fps responsive region which is localized between nucleotides -425 and -250 . This region contains four tandemly repeated SREs.

To determine if the four SREs contained within this region of the Egr-1 promoter were sufficient to confer v-Fps responsiveness, we cloned different segments of this region onto the heterologous herpes thymidine kinase minimal promoter as described in Materials and methods. pE425/250TKCAT (Figure 4a) contains the four distal SREs whose deletion diminished v-Fps-responsiveness of pEgr-1 P1.2. Activation of CAT gene expression by v-Fps from pE425/250TKCAT was comparable to that observed for pEgr-1 P1.2 and pE425 (Figure 4b). Similarly, sequential removal of the distal SREs from the PTK35CAT constructs diminished v-Fps responsiveness (Figure 4b). Thus, the SREs localized between -425 and -250 are not only necessary for induction of Egr-1 by v-Fps, but also sufficient.

v-Fps activates gene expression controlled by individual SREs contained within the v-Fps-responsive region of the Egr-1 promoter

The data presented in Figure 4, suggest that v-Fps-induced Egr-1 expression is mediated by SREs. We therefore, examined whether individual SREs from this region could respond to v-Fps. Several constructs were made containing individual SREs upstream from the HSV-TK promoter (Figure 5a). pTKCATSRE1 contains the most distal SRE from the v-Fps-responsive region. This SRE was able to confer v-Fps-responsiveness to the HSV-TK minimal promoter (Figure 5b). However, the level of conferred responsiveness was substantially lower than that conferred by the collection of four SREs that gave maximal v-Fps-responsiveness (Figure 5b). pTKCATSRE2 contained the second most distal SRE upstream from the HSV-TK promoter was also weakly responsive to v-Fps. pTKCATSRE1-2 which contains the two most distal SREs with the short intervening sequence, was more responsive than either of the two SREs by themselves. pTKCATSRE3 containing the third SRE, was also weakly responsive to v-Fps. pTKCATSRE4 gave little or response to v-Fps. Thus, the individual SREs in the v-Fps-responsive region were not as responsive to v-Fps the collection of SREs in this region were.

DISCUSSION

In this report, we have demonstrated that v-Fps induced expression of the mitogen-responsive transcription factor Egr-1 is a primary response to v-Fps that does not require protein synthesis. Deletion analysis of the Egr-1 promoter showed that v-Fps responsiveness was localized to a cluster of four CarG box-containing SREs located between -425 and -250 relative to the transcription start site. This region was sufficient to confer v-Fps-responsiveness to a heterologous promoter. Although, v-

Fig. 5. Individual SREs from the v-Fps-responsive region of the Egr-1 promoter confer v-Fps-responsiveness. NIH 3T3 cells were transfected with pIV2.3 and constructs containing individual SREs from the v-Fps-responsive region of the Egr-1 promoter fused to the pTK35CAT plasmid. a. Diagram of plasmids used to make pTKCATSRE1,2,3, and 4. b. CAT activity from cells transfected with pTKCATSRE constructs containing individual SREs in the presence of pIV2.3 was assayed and normalized to the amount of CAT activity generated in the presence of pIV2.3 and pTK35CAT. Data are the mean of at least four independent experiments.

Fps was able to activate individual SREs from the v-Fps responsive region of the Egr-1 promoter, the presence of a cluster of SREs was important for v-Fps-responsiveness. These data suggest that v-Fps transcriptionally activates Egr-1 via this cluster of SREs. We previously demonstrated that this cluster of SREs mediated responsiveness to the oncogenic protein tyrosine kinase v-Src. This may reflect common signaling mechanisms by protein-tyrosine kinases for activating the Egr-1 promoter. In this regard, it is important that the Egr-1 promoter contains other transcriptional control elements including two AP-1 sites. AP-1 sites are frequently activated by agonists of protein kinase C. Both v-Fps (4) and v-Src (4,37) activate protein kinase C-mediated intracellular signals. Thus, the activation of the Egr-1 promoter via SREs rather than AP-1 sites suggests a complex selectivity by the intracellular signals initiated by v-Fps and perhaps other protein-tyrosine kinases.

Serum, growth factors and phorbol esters have also been shown to induce Egr-1 SREs (38). Induction was shown to be mediated by serum response factor (SRF) (38), a factor that binds to C-ArG boxes contained within SREs (34, 35, 36). Thus, it is possible that v-Fps-induced Egr-1 involves the activation of SRF. The fourth SRE, by itself, was not responsive to v-Fps. Consistent with an SRF requirement for the v-Fps activation of Egr-1, the CARG box of SRE4, has only 5 A/Ts between the CC and GG of the CARG box and does not bind SRF (38). The differential activation of the remaining SREs within the Egr-1 promoter that do bind SRF suggests that sequences flanking the CARG boxes could be important for responsiveness to v-Fps and that additional factors may be involved. The intracellular components that contribute to the activation of SRF are not known; however SRF requires phosphorylation for induction of transcription (35); thus a protein kinase may be involved. Other v-Fps-induced factors that might activate, or function synergistically with SRF to induce Egr-1, remain to be determined.

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