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Separation of v-Src–induced Mitogenesis and Morphological Transformation by Inhibition of AP-1

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v-Src activity results in both morphological transformation and reentry of quiescent chick embryo fibroblasts (CEF) into cell cycle. We have previously used temperature-sensitive v-Src mutants to show that enhanced activity of cellular AP-1 in the first few hours after activation of v-Src invariably precedes the biological consequences. Here we have investigated whether the early activation of AP-1 is essential for any or all of the v-Src responses by using a mutant c-Fos that comprises the leucine zipper and a disrupted basic region. Expression of the c-Fos mutant partially reduced cellular AP-1 activity in exponentially growing cells. However, in CEF that had been made quiescent by serum deprivation, v-Src–induced stimulation of AP-1 DNA binding activity was substantially reduced. In addition, quiescent CEF stably transfected with this mutant show an impaired mitogenic response to v-Src, indicating that the AP-1 stimulation is a necessary prerequisite for cell-cycle reentry. The ability of v-Src to morphologically transform quiescent CEF was not impaired by the inhibition of AP-1 stimulation, indicating that the mitogenic and morphological consequences of v-Src have distinguishable biochemical mediators. Focal adhesion kinase, a recently identified determinant of cell morphology, undergoes a gel mobility shift, characteristic of its hyperphosphorylated state, in response to v-Src activation in cells expressing the inhibitory AP-1 protein. This provides further evidence that the pathways that regulate morphological transformation are independent of AP-1.

INTRODUCTION

The v-Src oncoprotein transforms fibroblasts in culture and can stimulate quiescent rat and chicken fibroblasts to reenter cell cycle (Bell *et al.*, 1975; Durkin and Whitfield, 1984; Welham *et al.*, 1990; Catling *et al.*, 1993). Recent evidence implies that v-Src initiates events at the cell periphery, primarily signaling protein interactions mediated by the Src homology (SH)-2 and SH3 domains (reviewed in Pawson and Schlessinger, 1993) and protein tyrosine phosphorylations, which in turn activate intracellular effector systems and give rise to altered gene expression in the nucleus. Although many parallels have been drawn between growth factor receptor– and v-Src–mediated signaling and putative mediators identified, the pathways by which v-Src transforms cells still remain to be elucidated. In particular, the specific events that distinguish the oncogenic stimulus provided by v-Src from the growth stimulus of growth factors are not well understood.

Key transcription factors are involved in coupling signals at the cell periphery to gene expression in the nucleus by binding to specific DNA target sequences in the promoters of responsive genes. AP-1 is one transcription factor involved in the conversion of signals provided by activated growth factor receptors, oncogenes, and protein kinase C into the altered gene expression that underlies the biological consequences of these stimuli. AP-1 is dimeric, comprising members of the Jun and Fos families. The first nuclear response to growth factor–induced mitogenic signaling is rapid induction of the "immediate-early" class of genes, which includes c-*fos* and c-*jun*, whose stimulation is important for exit from G_0 and cell-cycle progression. In addition to transcriptional stimulation, AP-1 DNA

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binding and transactivation activities can also be regulated by posttranslational mechanisms in response to biological stimuli, including oncogenic Src, Ras, and Raf (Abate *et al.*, 1990; Binetruy *et al.*, 1991; Boyle *et al.*, 1991; Frame *et al.*, 1991; Lin *et al.*, 1992; Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). We have previously examined the early regulation of AP-1 in resting CEF that are induced to reenter cycle and transform upon activation of temperature sensitive (*ts*) v-Src (Catling *et al.*, 1993). DNA binding and transactivation are stimulated soon after shift to permissive temperature, effects are mediated partly by enhanced transcription of c-*fos* and altered phosphorylation of c-Jun. A nonmyristylated *ts* v-Src protein does not stimulate AP-1 activity, is transformation-defective, and is unable to induce CEF to reenter cycle, implying that crucial signaling events at the membrane are required for the early increase in AP-1 activity that accompanies v-Src–induced mitogenesis and transformation. In rat fibroblasts AP-1 activity is also enhanced in response to activation of v-Src, although this is not mediated by increased synthesis of c-*fos* or c-*jun* transcripts (Welham *et al.*, 1990). Thus, stimulation of AP-1 correlates with v-Src activity in a number of settings, although achieved by different mechanisms, implying an important role for AP-1 regulated genes in the biological consequences of v-Src.

Dimerization of the components of AP-1 is mediated by the leucine repeats of Jun and Fos and is essential for DNA binding. Fos does not homodimerize and so does not bind to DNA independently of Jun, whereas Jun is able to form homodimers and heterodimers with Fos, the latter having greater stability and higher affinity for the specific DNA recognition sequence (reviewed in Gillespie, 1991). These properties of AP-1 have allowed the design of inhibitory mutants of Fos and Jun that are able to dimerize with their cellular partners but form nonfunctional complexes because of their inability to bind to DNA and lack of sequences normally associated with transactivation function. Such mutants have been used to demonstrate a role for AP-1 in transformation of chick embryo fibroblasts (CEF) by c-*jun* (Okuno *et al.*, 1991) and transformation of rat embryo cells by activated *ras* and deregulated c-*jun* and c-*fos* genes (Brown *et al.*, 1993).

In this present study we have examined the effects of expressing a mutated Fos protein on the mitogenic and morphological activities of v-Src. We conclude that AP-1 stimulation is required for mitogenesis but not the ultrastructural changes that give rise to the morphological appearance of v-Src–transformed cells. This implicates AP-1 as a nuclear target for the mitogenic pathway but not the cytoskeletal/morphological pathway initiated by v-Src and shows that these two pathways are, at least in part, biochemically separable.

MATERIALS AND METHODS

Cloning and Expression of c-fos Mutant with ts v-Src in CEF

The replication competent avian retrovirus (RCAN) expressing the *ts* mutant of v-Src, LA 29, was constructed as described previously (Catling *et al.*, 1993). Standard polymerase chain reaction (PCR) technology was used to obtain the basic and leucine zipper coding sequences of murine c-*fos* (amino acids 111–206) containing four codons that give rise to insertion of the amino acid sequence PLER (in the single letter amino acid code) at amino acid 161 in the murine c-Fos sequence. A Kozak consensus was incorporated into the 5′ primer to ensure efficient transcription initiation. *Hin*dIII and *Bam*HI sites were included in the 5′ and 3′ primers, respectively, to allow ligation with a *Bam*HI/*Eco* R1 fragment encoding the HE14 portion of the human estrogen receptor (ER) and the pSFCV-LE-Sa$^+$ vector (Fuerstenberg *et al.*, 1990) cut with *Hin*dIII and *Eco*RI (Figure 1A). The cloned PCR product was confirmed by sequencing. Plasmids used in cell transfection experiments were purified through caesium chloride gradients. Sequences encoding the HE14 portion of the human ER (Kumar *et al.*, 1986), preceded by an initiating methionine codon, were also cloned into pSFCV as control.

Primary CEF cultures were grown and cotransfected at low density with RCAN-Src construct (5–10 μg per 25-cm^2 flask) and pSFCV-δFos$_i$-ER fusion construct (10 μg per 25-cm^2 flask) at restrictive temperature (41°C) using the calcium phosphate method. Growth and transfection were as described in Crouch *et al.*, 1990 with the addition of 1 μM β-oestradiol. Transfected cultures were selected in G418 and found to uniformly express v-Src and the ER fusion proteins by immunoblotting using anti-Src monoclonal EC10 (Tissue Culture Services, UK) and the anti-ER monoclonal (F3-A) (Euromedex, Schiltighein, France) (Figure 1B).

Electrophoretic Mobility Shift Assay (EMSA)

Total cell protein extracts were prepared as described in Marais *et al.* (1993). Briefly, whole cell extracts were prepared from 90-mm dishes of cells. The cells were washed twice with phosphate-buffered saline (PBS) and lysed with 50 μl extract buffer. Extracts were sonicated and clarified by high speed centrifugation; protein concentration of supernatants was determined by the Bio-Rad Laboratories (Richmond, CA) protein assay kit and stored at −70°C. Ten micrograms of cell extract was incubated for 30 min on ice with a radiolabeled oligonucleotide containing the AP-1 consensus from the collagenase promoter. Incubation buffers and acrylamide gels were as described in Frame *et al.* (1991). Similar results to those found with AP-1 DNA binding activities from whole cell extracts were obtained using proteins extracted from purified nuclei (unpublished observations).

Mitogenesis

Low density cultures were made quiescent at restrictive temperature by incubating in 0.2% serum for 48–72 h. DNA synthesis was measured at various times after stimulation by pulse labeling with tritiated methylthymidine (Amersham, Arlington Heights, IL) as described previously (Welham *et al.*, 1990).

Protein Immunoblotting (Including Focal Adhesion Kinase [FAK] Detection)

Proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose using a semidry blot procedure. After appropriate blocking (3% Marvel or 3% bovine serum albumin for anti-phosphotyrosine blots with 0.2% Tween 20 [Sigma, St. Louis, MO]) overnight at 4°C, blots were incubated with the primary antisera, washed extensively with PBS/0.2% Tween 20, and incubated with 1:5000 horse radish peroxidase–conjugated anti-mouse or anti-rabbit serum (Amersham). Reactive

proteins were visualised using enhanced chemiluminesence according to the manufacturer's instructions (Amersham). Primary antisera were used at the following concentrations: anti-Src EC10, 1:5000; anti-ER, 1:1000; anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), 1:5000.

CEF protein lysates for immunoprecipitation were prepared in RIPA buffer (Black *et al.*, 1991) as described previously (Catling *et al.*, 1993). Before immunoprecipitation, lysates were clarified by centrifugation at 14 000 rpm in a Sorvall SS34 rotor (Newton, CT) for 20 min at 4°C, precleared with 10 μl of normal rabbit serum and protein A-sepharose (Sigma). FAK was immunoprecipitated from 300–500 μg protein by incubating with 2 μl anti-FAK (Upstate Biotechnology) overnight on ice. Immunoprecipitated FAK was collected on protein A-sepharose beads coated with rabbit anti-mouse IgG. Proteins were resolved by 7.5% SDS-PAGE, immunoblotted, and probed with anti-phosphotyrosine IgG.

RESULTS

Cloning and Expression of the c-fos Mutant

We have used an approach similar to that of other groups to inhibit AP-1 by expression of an interfering mutant of c-Fos. The mutant was designed to bind to its cellular partners from the Jun family of proteins but form dimers that could not bind to the AP-1 recognition sequence. We made use of the published observations that the leucine zipper and DNA binding domains were sufficient to specify dimerization and DNA contact in the absence of the rest of the proteins (Abate *et al.*, 1990). Thus, a region of c-*fos* encoding the entire leucine zipper and basic DNA binding domain, which contained a four amino acid helix disrupting insertion between the basic region and leucine zipper, was cloned into SFCV (δFos_i) (described in MATERIALS AND METHODS). Insertion of codons for the same four amino acids into c-*fos* coding sequences has been shown to disrupt Fos function and inhibit cellular AP-1 in a dominant manner (Yoshida *et al.*, 1989, Okuno *et al.*, 1991). The c-*fos* sequences were fused to the HE14 portion of the human ER (Figure 1A), providing an epitope tag. As control, the HE14 domain preceded by an initiating methionine codon was also introduced into SFCV (mER) (Figure 1A). These constructs were cotransfected into CEF with the replication competent retroviral vector RCAN encoding the *ts* v-Src mutant LA29. After selection with G418, the cell lysates were probed with Src- and ER-specific antibodies. As expected, the cells were uniformly infected with the v-Src–expressing retrovirus (Figure 1B, lanes 6 and 7). Cells transfected with the δFos_i-ER and mER constructs expressed proteins of the expected molecular weights that reacted with the ER antibody (Figure 1B, lanes 2 and 3, respectively). Cells transfected with δFos_i-ER also consistently produced proteins with lower apparent molecular weights, perhaps indicating cleavage within the cell (Figure 1B, lane 3). The δFos_i-ER and mER coding sequences were subcloned into a transcription vector and transcribed and translated in vitro, both constructs giving rise to translation products of the predicted size (unpublished observations). The hormone responsive sequences from the ER are frequently used to confer oestradiol-dependent conditionality on heterologous protein function. One micromolar β-oestradiol was therefore routinely added to the culture medium with no effect on cell growth rate or viability. We have no evidence that the function of the c-Fos mutant was made conditional by the ER fusion.

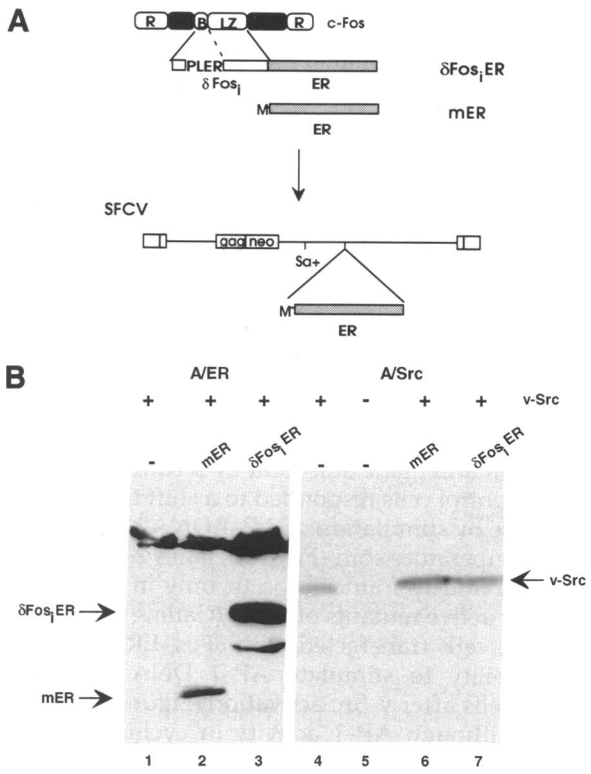

Figure 1. (A) Construction of pSFCV-mER and pSFCV-δFos_i-ER. The murine c-*fos* basic region (B) and leucine zipper (LZ) coding sequences, containing a four-amino-acid PLER insertion, were isolated by PCR and cloned into the pSFCV-Sa$^+$ avian retroviral vector as described in MATERIALS AND METHODS. Coding sequences for the HE14 portion of the human ER were also cloned into pSFCV as control (mER). Other functional domains of c-Fos shown are the regulatory regions (R) and regions required for trans-activation (■). (B) Expression of mER and δFos_i-ER fusion proteins with *ts* v-Src. Extracts of growing CEF were prepared and immunoblotted using anti-ER (A/ER) (lanes 1–3) or EC10 (A/Src) (lanes 4–7). Three hundred micrograms protein from CEF-expressing SFCV vector alone (lane 1), mER (lane 2), or δFos_i-ER (lane 3), all coexpressing *ts* v-Src, were separated by SDS-PAGE, blotted, and probed with A/ER. Arrows show the species that comigrated with the primary translated products when translated in vitro. One hundred micrograms protein from CEF-expressing *ts* v-Src (lanes 4, 6, and 7), or RCAN vector alone (lane 5), with SFCV vector alone (lanes 4 and 5), mER (lane 6), or δFos_i-ER (lane 7), were immunoblotted using A/Src. v-Src protein is shown by the arrow.

AP-1 Inhibitory Properties of the c-fos Mutant

Cells cotransfected with the mER or δFos_i-ER constructs and RCAN-*ts* v-Src were characterized with respect to

their endogenous AP-1 DNA-binding activity. Extracts of cells growing exponentially were prepared and tested for their ability to bind to a labeled oligonucleotide containing a single AP-1 site using EMSAs. A decrease in AP-1 DNA-binding activity relative to the mER control cells was consistently observed with cells expressing δFos$_i$-ER (Figure 2, lane 2), although there always remained appreciable AP-1 DNA-binding activity. The specificity of the AP-1 DNA-binding complex was confirmed by inhibition with unlabeled competitor (Figure 2, lanes 3 and 4). Thus, expression of δFos$_i$-ER resulted in a partial inhibition of cellular AP-1 activity in growing CEF that had no obvious effect on cell growth.

We next examined the ability of activation of *ts* v-Src to stimulate AP-1 DNA binding in CEF expressing mER and δFos$_i$-ER and made quiescent by serum deprivation. The mER control cells responded to a shift to permissive temperature by stimulation of AP-1 DNA binding, seen 1 h after temperature shift (Figure 3, lanes 2–4), an effect that we previously found to occur only in response to biologically active mutants of v-Src (Catling *et al.*, 1993). In contrast, cells transfected with δFos$_i$-ER exhibited a reduced ability to stimulate AP-1 DNA binding in quiescent cells after v-Src activation (Figure 3, lanes 6–8). Thus, although AP-1 activity in cycling cells was only partially inhibited by δFos$_i$-ER, the ability of v-Src to stimulate AP-1 in quiescent cells was suppressed to a considerably greater extent.

The Effect of the c-fos Mutant on v-Src–induced Mitogenesis

CEF expressing *ts* v-Src with the control mER or mutant Fos protein were made quiescent as described above and either unstimulated (Figure 4A) or stimulated either by shift to permissive temperature in the absence of additional factors (Figure 4B) or by the addition of newborn calf serum (5%) (Figure 4C). During the course of the experiment, the levels of thymidine uptake for both transfectants remained low and invariant in the absence of any stimulus (Figure 4A). In the mER control cells, activation of v-Src and serum addition resulted in entry into S-phase as measured by uptake of tritiated thymidine (Figure 4, B and C). Cells expressing δFos$_i$-ER exhibited a reduced capacity to reenter cell cycle in response to either addition of serum or temperature shift (Figure 4, B and C). The extent of inhibition of mitogenesis in response to v-Src and serum varied between batches of CEF prepared at different times and depended on them being properly quiescent after serum deprivation.

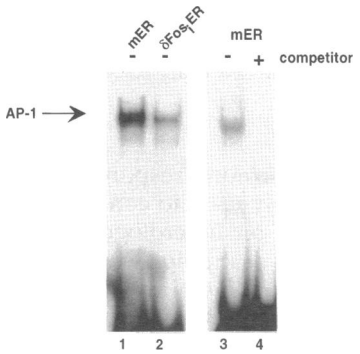

Figure 2. AP-1 DNA-binding activity in growing CEF. EMSAs were carried out as described in MATERIALS AND METHODS using a labeled collagenase AP-1 probe and extracts from CEF-expressing mER or δFos$_i$-ER (lanes 1 and 2, respectively). Competition with 100-fold excess unlabeled homologous competitor demonstrated specificity (lanes 3 and 4). Retarded AP-1 probe is indicated by the arrow.

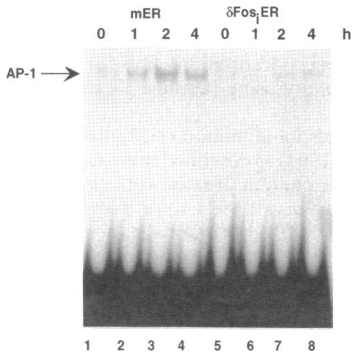

Figure 3. Inhibition of AP-1 stimulation in response to activation of *ts* v-Src. EMSAs carried out using extracts prepared from CEF-expressing mER (lanes 1–4) or δFos$_i$-ER (lanes 5–8) that had been made quiescent and stimulated by shift to permissive temperature for 1 h (lanes 2 and 6), 2 h (lanes 3 and 7), and 4 h (lanes 4 and 8), respectively.

Morphological Transformation Is Unaffected by AP-1 Inhibition

To address the role of AP-1 stimulation in the v-Src–mediated morphological changes, CEF coexpressing the c-Fos mutant protein with *ts* v-Src were made quiescent at restrictive temperature and either retained at restrictive temperature or shifted to permissive temperature for 24–36 h. At 41°C all cultures were flat with the typical appearance of normal CEF (Figure 5, A and B). After shift to 35°C, the morphological appearance of the cultures stably transfected with mER or δFos$_i$-ER was identical, with both exhibiting the refractile irregular features of v-Src–transformed CEF (Figure 5, C and D). Thus, AP-1 stimulation is not required for the morphological aspects of v-Src–induced transformation, implying that the mitogenic and morphological consequences of v-Src activity are biochemically separable.

FAK Activation by v-Src Is Unaffected by AP-1 Inhibition

p125 FAK, a recently identified nonreceptor protein tyrosine kinase present in cellular adhesion plaques that

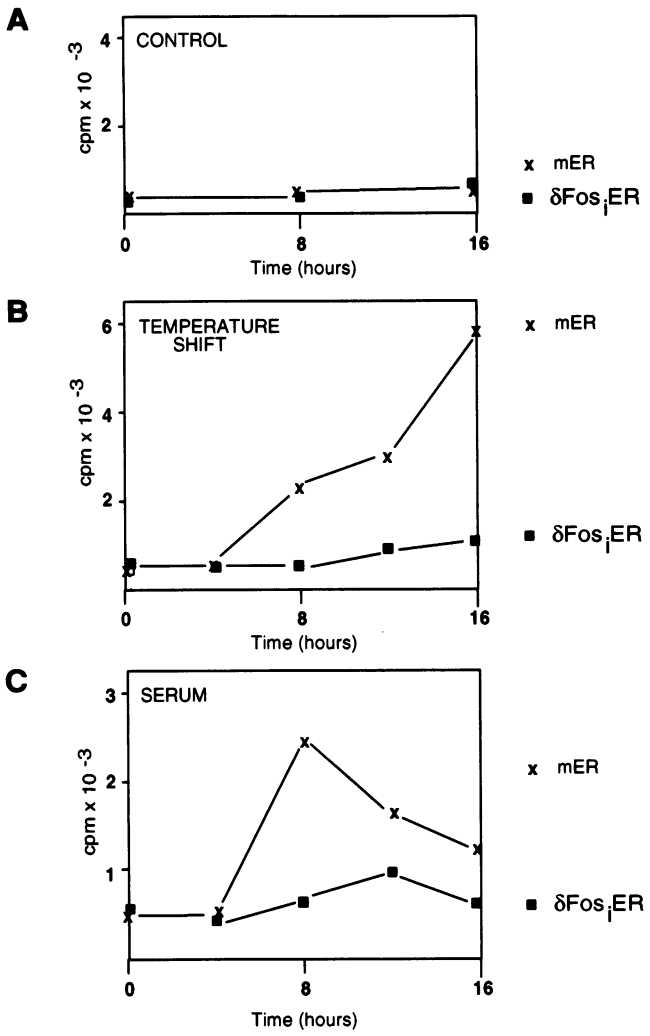

Figure 4. Effect of c-Fos mutant on mitogenesis. Thymidine uptake of CEF coexpressing *ts* v-Src with mER or δFos$_i$-ER that had been made quiescent by serum deprivation at restrictive temperature and either retained at restrictive temperature (A, CONTROL), shifted to permissive temperature (B, TEMPERATURE SHIFT), or provided with 5% newborn calf serum (C, SERUM).

is itself activated by tyrosine phosphorylation, is implicated in mediating the cytoskeletal changes that give rise to the morphological appearance of transformed cells (Schaller *et al.*, 1992). Thus, we examined tyrosine phosphorylation of p125 FAK in response to activation of *ts* v-Src in CEF transfected with the inhibitory δFos$_i$-ER construct. FAK was immunoprecipitated from cell lysates, the proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. In quiescent cells coexpressing v-Src with mER at restrictive temperature (Figure 6, lane 2), tyrosine phosphorylation of FAK was considerably above that in cells not expressing v-Src (control RCAN vector) (Figure 6, lane 1). In response to shift to permissive temperature for 24 h, FAK appeared to undergo a gel mobility shift to an apparently higher molecular weight (Figure 6, lane 3), presumably as a result of hyperphosphorylation and/or a conformational change. These observations are in agreement with previous reports that increased phosphorylation of p125 is characterized by a shift in gel mobility in cells transformed by activated variants of c-*src* and v-*src* (Kanner *et al.*, 1990; Seidel-Dugan *et al.*, 1992). We are not able to conclude whether the total phosphotyrosine content of p125 was increased in response to activation of v-Src in these experiments because the immunoblots were not titrated. In CEF transfected with δFos$_i$-ER, the change in gel mobility of FAK in response to activation of v-Src was similar to the mER control (Figure 6, lanes 4 and 5), indicating that the posttranslational alteration of FAK that gives rise to the change in migration on SDS-PAGE does not require the early stimulation in AP-1 activity that normally accompanies v-Src activation.

DISCUSSION

In this study we have used a mutant of c-*fos* encoding a protein that interferes with the normal regulation of the cellular transcription factor AP-1 to examine the need for AP-1 in v-Src–induced mitogenesis and morphological transformation. Stable expression of the c-Fos leucine zipper and disrupted basic region, fused to the HE14 portion of the human ER, substantially reduced the early v-Src–induced stimulation of AP-1 in cells made quiescent by serum deprivation but not in cells growing exponentially. This AP-1 reduction was accompanied by a marked impairment of v-Src–driven exit of quiescent cells from G_0.

These observations parallel closely those of Nishikura and Murray (1987), who showed that NIH3T3 cells expressing antisense *fos* cycled almost normally in exponential growth but were unable to leave G_0 and reenter cycle in response to platelet-derived growth factor. An antisense approach has also demonstrated a need for AP-1 in the mitogenic response of target cells to tumour necrosis factor-α (Brach *et al.*, 1993). These observations, with ours, indicate that AP-1 stimulation is a general prerequisite for the transition from G_0 to G_1, although this early AP-1 activation is not sufficient for cells to enter S-phase in the absence of other factors, because there is a second requirement for v-Src activity in mid-G_1 (Wyke *et al.*, 1993).

We also found that in CEF transfected with dominant negative Fos the morphological changes characteristic of v-Src transformation were unimpaired, showing that AP-1 activation is a specific and necessary nuclear consequence of v-Src activity that is dispensable for morphological transformation. This finding is ostensibly at variance with the conclusions of some previous work. Expression of antisense *fos* RNA partially inhibits *ras*-induced transformation (Ledwith *et al.*, 1990), yet an-

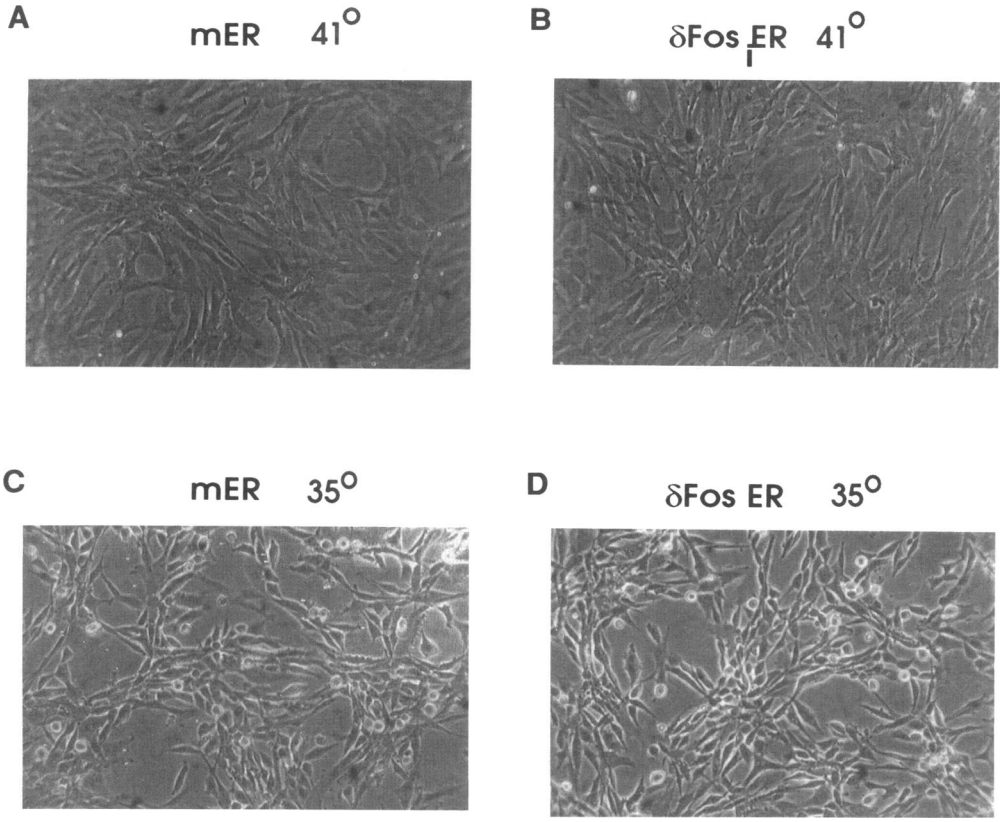

Figure 5. Morphology of quiescent CEF cultures expressing mER (A and C) or δFos_1-ER (B and D) at restrictive (A and B) and permissive (C and D) temperatures.

tibodies to p21 Ras inhibit v-Src transformation (Smith *et al.*, 1986), implying that Ras function is needed for v-Src to transform cells. In addition, several groups (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Okuno *et al.*, 1989; Ransone *et al.*, 1990; Smeal *et al.*, 1989; Turner and Tjian, 1989) have reported derivatives of both c-Fos and c-Jun that act as transdominant inhibitors of AP-1 of the kind that have been shown to suppress oncogene-induced transformation. In particular, a c-*jun* dominant negative mutant suppressed transformation of rat embryo cells induced by oncogenic *ras* or deregulated c-*jun or c-fos* (Brown *et al.*, 1993). This is consistent with other reports of Jun and Fos proteins interfering with *ras* transformation (Lloyd *et al.*, 1991; Wick *et al.*, 1992), although in the latter case the inhibition may have been by mechanisms other than direct reduction in AP-1-dependent transactivation. The consensus that v-Src requires Ras activity and that the latter acts through AP-1 can only be partially reconciled with our finding that AP-1 is dispensable for v-Src transformation by postulating that Ras mediates aspects of v-Src activity independently of AP-1. These AP-1-independent activities must be insufficient for Ras transformation but adequate for transformation by v-Src, possibly because v-Src has other Ras- and AP-1-independent effects.

Our conclusion that v-Src–induced mitogenesis and transformation are at least partly separable, although not predicted by the findings discussed above, are, in fact, supported by several earlier observations. Nonmyristylated v-Src mutants were nontransforming but promoted proliferation of neuroretinal cells (Calothy *et al.*, 1987), although the same mutants were nonmitogenic for quiescent CEF as used in this study (Catling *et al.*, 1993). In addition, some *ts* mutants dissociated morphological changes from growth-related parameters such as cell density and serum- and anchorage-independent multiplication (Weber and Friis, 1979). The correlates of mitogenesis used in these studies were, however, relatively poorly understood and cannot be readily equated with the transit out of G_0 that we have examined. Closer parallels for our findings were the observations of Beug *et al.* (1978), who reported that *ts* v-Src mutants can transform enucleated cells, and several studies showing that transformation by *ts* mutants occurred when de novo transcription was suppressed by Actinomycin D (Kawai and Hanafusa, 1971; Bader, 1972; Biquard and Vigier, 1972). Such work clearly showed that nuclear functions are not required for some effects of v-Src at the cell periphery, but the indiscriminate ablation of nuclear activity precludes both the prolonged observation and further dissection of the phenomenon that are permitted by our approach.

Our initial observations on FAK exemplify this potential for further analysis. FAK, a protein tyrosine kinase present in cellular adhesion plaques, is believed to

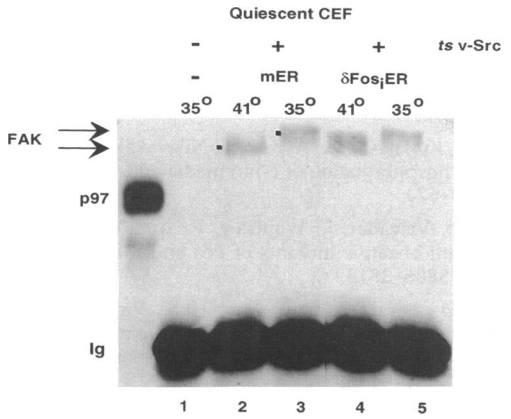

Figure 6. Activation of FAK in response to activation of *ts* v-Src. FAK was immunoprecipitated from CEF-expressing RCAN and SFCV vectors as control (lane 1), CEF coexpressing *ts* v-Src with mER (lanes 2 and 3), or δFos$_i$-ER (lanes 4 and 5), separated by SDS-PAGE, blotted, and probed with anti-phosphotyrosine. Before harvesting for immunoprecipitation, the cells had been made quiescent by serum deprivation at the restrictive temperature of 41°C and maintained at this temperature (lanes 2 and 4) or shifted to the permissive temperature of 35°C for 18 h. The faster and slower migrating forms of FAK are shown by the arrows. The 97-kDa molecular weight protein marker and the anti-FAK immunoglobulin (Ig) present in the immunoprecipitate are also shown.

play an important role in the ultrastructural changes that occur at the G_2-M transition during the cell cycle, when c-Src is active, and in response to the v-Src–transforming protein (reviewed in Zachary and Rozengurt, 1992; Schaller and Parsons, 1993). p125 FAK is a cytoplasmic target for oncogenically active forms of Src that result in elevated FAK tyrosine phosphorylation and activity (Kanner *et al.*, 1990). A complex between FAK and Src, both members of the nonreceptor class of protein tyrosine kinases, is mediated by the Src SH2 domain and the major site of autophosphorylation in FAK (Schaller *et al.*, 1994). Activation of FAK and/or translocation of active Src to focal adhesions may lead to enhanced tyrosine phosphorylation of other components of focal adhesions, for example, vinculin and paxillin, both known to exhibit increased tyrosine phosphorylation in v-Src–transformed cells (Sefton *et al.*, 1981; Glenney and Zokas, 1989). The lack of effect of AP-1 inhibition on the v-Src–mediated effects on FAK implies that its hyperphosphorylation, and presumed activation, is independent of this early nuclear consequence of v-Src activity. However, it is not yet known whether FAK itself provides a signal that is transmitted to the nucleus and alters expression of genes involved in uncontrolled growth or other tumor-associated properties, such as invasion or angiogenesis. It seems likely that, although v-Src mitogenic and morphological effects are partially separate in vitro, full transformation in vivo will need cell growth and hence the activation of both pathways. These concepts are in keeping with a recent detailed study by Miao and Curran (1994) on the kinetics of transformation by c-*fos* that indicated that, although AP-1 function may contribute to the transforming activities of *src*, c-Fos alone cannot be fully responsible for all aspects of the transformed phenotype. The ability to ablate specific nuclear consequences of v-Src activity will permit dissection of the interplay between this oncoprotein's mitogenic and morphological transforming properties.

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