Mitogenesis by v-Src: Fluctuations throughout G₁ of Classical Immediate Early AP-1 and Mitogen-activated Protein Kinase Responses That Parallel the Need for the Oncoprotein

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Abstract
Activation of the tyrosine kinase of a temperature-sensitive mutant v-Src oncprotein in quiescent Rat-1 cells leads to passage through the cell cycle. Temperature shift experiments show that v-Src is needed to leave G₀ to pass a relatively stable G₁ "pause" point, and to pass a later G₁ point committing cells to S phase. Classic immediate early responses that activate both AP-1 DNA binding and mitogen-activated protein (MAP) kinase are induced at G₀ exit, but unexpectedly they rise again in mid-G₁ and before the onset of S phase, fluctuations that parallel the need for v-Src. An estrogen-inducible mutant c-Raf-1 renders these cells susceptible to mitogenic stimulation by β-estradiol, without v-Src activity, but greatly inhibits the ability of v-Src to induce DNA synthesis and MAP kinase, probably because v-Src physically associates with inactive c-Raf-1 at permissive but not restrictive temperature. This implicates c-Raf-1 association with enzymically active v-Src and consequent activation of the MAP kinase pathway in v-Src mitogenesis. Furthermore, temperature shift experiments indicate that the mid-G₁ peak of MAP kinase activity is associated with cells reaching the G₁ pause point, while the pre-S phase peak is needed for DNA synthesis. In contrast, cell transformation by v-Src does not require enhanced MAP kinase activity at any stage of the cell cycle.

Introduction
The induction of neoplasia by v-Src, the prototype for non-receptor protein tyrosine kinase oncproteins, comprises changes in both cell growth and cell behavior. Activation of a temperature-sensitive (ts) v-Src gene in cells in culture leads, in several hours, to altered cellular shape and motility, and if the cells have been arrested in the G₀ phase of the cell cycle by serum deprivation, they are concomitantly driven through the cycle and into mitosis (1, 2). The rapid reversibility of ts v-src mutants enables the effect of pulses of v-Src activity to be analyzed, and these have revealed periods during which the oncprotein is needed for mitogenesis of G₀-arrested Rat-1 cells (3). The first v-Src requirement is in leaving G₀, and it is associated with a transient increase in activity of the AP-1 transcription factor in both Rat-1 and chick cells (2, 4). AP-1 functioning is not needed for morphological transformation of chick cells by v-Src, but it is essential for their mitogenic response (5). This early AP-1 increase is not, however, sufficient for v-Src mitogenesis, because in Rat-1 cells, v-Src is also required later in G₁ for successful DNA synthesis.

If cells in G₀ are exposed to a pulse of v-Src activity for several hours, a significant proportion reach a relatively stable pause point in G₁, from which they undergo accelerated progression to S phase upon reactivating v-Src (the remaining cells stay in, or return to, a point temporally indistinguishable from G₀). The v-Src requirement for cells at this pause point to progress to S phase is of only a few hours duration, because once cells are within about 6 h of the onset of DNA synthesis, they pass a point of commitment and can then proceed through S phase and mitosis without v-Src activity (3). There are thus three points at which v-Src activity is essential in the preparation for S phase: exit from G₀, transit of the pause point, and transit of the commitment point.

The biochemical bases for these v-Src requirements, particularly in the second half of G₁, have not been defined. It has been reported, however, that Ras functions are needed in late G₁ to permit serum-induced mitogenesis and transformation by tyrosine kinase oncogenes (6). In addition, Ras GTP and MAP kinase levels rise in epithelial cells that are released from a G₁ block imposed by transforming growth factor β1 (7). A recent consensus proposes that v-Src mediates many of its effects through the signaling pathway that links Ras to MAP kinases and their various targets (8–12). In support of this concept, microinjection of Ras antibodies, or of dominant negative mutant Ras or MAP kinase kinase proteins, suppresses DNA synthesis and causes transient reversion of the altered morphology of v-Src-transformed NIH3T3 cells (6, 11, 13), while constitutive expression of MAP kinase kinase is sufficient to transform these cells (11, 12). Since one of the consequences of activating this pathway is to elevate AP-1 function (reviewed in Ref. 14), we set out to examine whether the mitogenic need for v-Src in the second half of G₁ involves the MAP kinase pathways and, like the earlier v-Src requirement, the activation of AP-1.

We show here that activation of ts v-Src in quiescent Rat-1 cells activates both AP-1 DNA binding and MAP kinase in an oscillating pattern, the peaks of activity

Received 6/28/95; revised 8/1/95; accepted 8/4/95.

1 This work was supported by the Cancer Research Campaign.
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The abbreviations used are: MAP kinase; mitogen-activated protein kinase; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; ts, temperature sensitive; NCBS, newborn calf serum.
approximating the previously defined temporal requirements for v-Src function in G1.

Results

Activation of AP-1 by v-Src. When quiescent Rat-1 cells bearing a single copy of the Rous sarcoma virus ts LA29 v-src gene are shifted to permissive temperature, there is an early increase in AP-1 DNA binding activity that peaks at 1–2 h and then declines (2, 3). These events are not merely independent of v-Src activity, about 8–14 h after exit from G0, with DNA synthesis starting at about 16–20 h and peaking 4–8 h later (3). To ask whether these later events that occur in AP-1 DNA binding, whole-cell extracts were prepared at frequent intervals throughout v-Src–induced G1 and S phases and tested for their ability to retard AP-1 oligonucleotide representing the collagenase 12-O-tetradecanoylphorbol-13-acetate response element (Fig. 1a). High levels of AP-1 DNA binding were found during G1 and S phases, but they showed fluctuations, with maxima at 6 h, 16 h, and 24 h in S phase. Binding at the 16- and 24-h peaks, like that at the peak upon exit from quiescence (2, 3), was not detectable by AP-1 oligonucleotide (Fig. 1b).

A faster migrating complex was also seen in quiescent Rat-1 ts LA29 cells, which did not change in response to v-Src activation, and in contrast to the bona-fide AP-1 complex, it was only weakly detectable by cold AP-1 oligonucleotide (Fig. 1a and b). The amounts of DNA binding in G1 were greater than those seen at exit from G0, and at the short exposures necessary to show the later fluctuations, a peak on leaving G1 is not very apparent (Fig. 1a). This early rise is, however, clearly seen with proteins from nuclear extracts, as shown in Fig. 2c and by Welham et al. (2) and Wyke et al. (3).

Mechanism of AP-1 Activation. To explore why oscillations of AP-1 DNA binding should occur in the presence of sustained v-Src activity, we examined the mechanism of activation throughout G1. We have shown previously (2) that the early enhancement of AP-1 activity by v-Src activation in quiescent Rat-1 ts LA29 cells is not accompanied by increased levels of c-fos and c-jun RNAs (which encode AP-1 components), even in the presence of inhibitors of protein synthesis. Examination of c-fos transcripts by RNA blotting throughout G1 showed that there was also no increase of c-fos RNA levels associated with later, more major, peaks of AP-1 DNA binding (Fig. 2a). Moreover, levels of c-Jun protein, detected by immunoblotting, were also invariant in quiescence and throughout G1 and S phases (Fig. 2b). These data suggested that the changes in AP-1 DNA binding were due to posttranslational mechanisms and the failure of the protein synthesis inhibitor, emetine, to abrogate the early enhancement of AP-1 DNA binding by v-Src (Fig. 2c) supported this (toxicity of emetine made it difficult to examine its effects at later stages of G1).

It remains possible that the later fluctuations of AP-1 activity involve proteins other than the AP-1 components, Jun and the Fos family members. However, antibodies to Fos family members and specific to c-Jun had the same effects on the gel retardations produced by protein extracts at 6, 16, and 24 h after v-Src activation as on protein from quiescent cells (Fig. 3a; data for 6-h extract not shown). Moreover, when the complexes were cross-linked to retarded DNA by UV light, the bands excised and the protein constituents resolved in a second dimension, the AP-1 complexes were found to consist predominantly of M, 39,000 and M, 55,000 subunits at all time points, presumably corresponding to c-Jun and to a Fos-related protein of similar size to c-Fos cross-linked to DNA (Fig. 3b). The major difference from quiescent cells in those stimulated by v-Src for 16 and 24 h is an increase in the DNA binding capacity of the c-Jun protein (compare Fig. 2b with Fig. 3b). Thus, by several criteria, the quantitatively different peaks of AP-1 DNA binding arise by qualitatively similar posttranslational mechanisms.

![Fig. 1. Gel retardation analyses of AP-1 DNA binding activity in whole-cell lysates after activation of ts v-Src in quiescent Rat-1 ts LA29 cells. a, time course of induction after shift to permissive temperature. b, analysis in the presence and absence of 100-fold excess competitor oligonucleotide of extracts from quiescent cells and of cells from 16- and 24-h peaks of activity. ■, the position of the minor retard that is only partially competent and is unresponsive to v-Src.](image-url)
Activation of MAP Kinase by v-Src. In response to the evolving consensus that the MAP kinase pathway is a major mediator of v-Src signaling and that AP-1 activity can be elevated by this pathway (see "Introduction"), we analyzed MAP kinase activation in cells impelled into cycle by continuous activation of v-Src (Fig. 4a). Levels of MAP kinase protein do not change significantly between quiescent cells and those traversing the cell cycle (data not shown), but there is an immediate and transient increase in MAP kinase activity as cells leave G1, measured by phosphorylation of MBP by immunoprecipitates with a p42 ERK2 MAP kinase antiserum. A second, broad and more substantial peak of activity occurs in mid-G1 (between 6 and 12 h in the example shown), with a third peak just before the onset of S phase, and further oscillations of activity in S (as with AP-1 responses, the significance of changes during S phase are difficult to assess because increasing cell asynchrony with time makes it possible that some cells are entering a new G1 phase). Although the length of the G1 phase varied slightly from experiment to experiment, we found that these fluctuations in MAP kinase activity paralleled, but did not necessarily coincide with, those in AP-1 DNA binding, and their apparently triphasic nature corresponded to the three defined requirements for v-Src activity (see "Introduction").

Regulation of MAP Kinase during v-Src Mitogenesis. As with changes in AP-1 DNA binding, continuous v-Src activation led to discontinuous peaks of MAP kinase activity, suggesting that the latter is subject to overriding cellular regulatory mechanisms. It is possible that this regulation does not need continuous v-Src activity but is set in train by exit of cells from G0. On the other hand, since there is a
correspondence between these peaks of MAP kinase activity and the need for v-Src during G₈ transit, the two events may be coupled.

To examine this, quiescent Rat-1 ts LA29 cells were exposed to an 8-h “pulse” of v-Src, which we have shown previously to be insufficient for DNA synthesis but adequate for a significant number of cells to reach the pause point (3). As shown in Fig. 4b, the 8-h pulse of v-Src is, indeed, insufficient for S-phase entry, and only the first and second peaks of MAP kinase activation are observed. Indeed, the second peak is abrupt, with shift to restrictive temperature at 8 h leading to an immediate decline in MAP kinase activity. Moreover, when duplicate cultures exposed to an 8-h pulse of v-Src were shifted back to permissive temperature at 16 h, there was a smaller 24-h peak of DNA synthesis than in cultures continuously exposed to v-Src (Fig. 4, compare c and a), showing that some but not all cells had, indeed, paused in G₈. DNA synthesis by these cells coincided with a marked rise in MAP kinase activity, specific to and commencing about 4 h after the reactivation of v-Src. We have
shown previously (3) that cells that had failed to reach or stay at the pause point were in a state temporally indistinguishable from Go, but this delayed onset of MAP kinase activity shows that there are, in fact, no cells remaining in a state equivalent to Go, and further indicates that an immediate increase in MAP kinase activity in response to v-Src is specific to G1 exit.

Role of the MAP Kinase Pathway in v-Src Mitogenesis

MAP kinase activities rise at, or shortly after, times at which v-Src activity is needed for G1 transit (Fig. 4a; Ref. 3), while MAP kinase and v-Src activities are closely coupled in temperature pulse experiments (Fig. 4, b and c). These findings suggest that MAP kinase may be a necessary and even sufficient mediator of v-Src mitogenesis, and we reasoned that an inducible activation of the MAP kinase pathway, independently of v-Src, would enable us to determine whether it could substitute for any of the v-Src needs during G1 progression.

An estrogen-inducible, amino terminally truncated, oncogenic Raf-1 construct, hARaf (Ref. 15; now termed ΔRaf-1:ER) was introduced into Rat-1 ts LA29 cells. In pools of ΔRaf-1:ER-containing cells and in a cell clone, R29RER-3, expressing low levels of the protein (Fig. 5a), there was no MAP kinase activation and no DNA synthesis in response to treatment with 1 μM β estradiol (Fig. 5b). Others have also found that Raf expression in Rat-1a cells, unlike 3T3, does not activate MAP kinase (15, 16). However, in a clone of Rat-1 ts LA29 cells selected for high level ΔRaf-1:ER expression (R29RER-8; Fig. 5a), β estradiol induction in quiescent cells efficiently led to MAP kinase activation and DNA synthesis independently of v-Src (Fig. 5b), as well as marked morphological transformation (data not shown), the latter as reported by Samuels et al. (15). As with v-Src, the MAP kinase activities were sustained through G1, but in an apparently fluctuating pattern.

An appropriate stimulus could thus activate MAP kinase and induce DNA synthesis in R29RER-8 cells. However, v-Src was an inefficient mitogen in these cells in comparison with parental Rat-1 ts LA29 cells in the absence of β estradiol (Fig. 6a). The diminished mitogenic response was accompanied by reduced MAP kinase activation (Fig. 6b), together rendering R29RER-8 cells unsuitable for examining the ability of MAP kinase functions to substitute for those of v-Src (note that DNA synthesis in the experiment shown in Fig. 6 starts about 4 h earlier than in Fig. 4 and in Rat-1 ts LA29 cells there is a consequent apparent compression of the mid and late G1, peaks of MAP kinase activity, although the pattern of elevated but fluctuating activity throughout G1 is evident in this, as in many repeat experiments). Samuels and McMahon (17) have shown that stimulated ΔRaf-1:ER expression inhibited Raf-1 activation and mitogenesis by platelet-derived growth factor and epidermal growth factor, but our findings suggest that this dominant negative effect can also occur in the absence of a β estradiol stimulus. This might be explicable if any ability of v-Src to signal through c-Raf-1 (18) is diminished by association with inactive ΔRaf-1:ER. When v-Src immunoprecipitates from R29RER-8 grown in the absence of β-estradiol were probed with an antisem to the estrogen receptor moiety of ΔRaf-1:ER, this latter protein indeed coprecipitated with v-Src (Fig. 7a). The association of ΔRaf-1:ER with v-Src was, moreover, temperature sensitive, either because LA29 v-Src looses poorly from the plasma membrane at restrictive temperature (19) or because the association requires an active v-Src kinase. It is noteworthy that Marais et al. (18) find that nonmyristoylated Src, which cannot locate at the plasma membrane, cannot activate c-Raf-1.

These findings show that v-Src mitogenesis depends on normal Raf-1 functions, strongly implicating the Raf-1/MAP kinase pathway in this process. This pathway is not, however, needed for other aspects of v-Src function, since v-Src activation efficiently transformed R29RER-8 cells (Fig. 7b), thus uncoupling the requirements for v-Src mitogenesis and transformation.

Discussion

Activation of a single ts v-src gene in quiescent Rat-1 cells induces synchronous G1 transit and a sharp wave of DNA synthesis (Refs. 2 and 3; Fig. 4a). At the peak of thymidine incorporation, 24 h after v-Src activation, about one-half the cells are in S phase as judged by 5-bromo-2′-deoxyuridine labeling (2) or fluorescence-activated cell sorting (3), with cells almost doubling in number thereafter (3). The fidelity of this response, coupled with the rapid reversibility of the v-Src tyrosine kinase, has allowed us to show that v-Src is needed both at exit from Go and at points during G1 to overcome checks during progression to S phase. In this study, we have further exploited this uniquely noninvasive way of efficiently punctuating G1 progression in mass populations of cells to examine some of the concomitants of these v-Src requirements. We find that both AP-1 DNA binding and p42 ERK MAP kinase activities fluctuate throughout G1 in patterns that parallel the needs for v-Src, with peaks upon leaving Go in mid G1, and just before the onset of S phase.

Enhancement of AP-1 DNA binding and MAP kinase activities are classical immediate early events in cells leaving the G0 state, but these activities have also been found elevated at other stages of the cell cycle in a number of reports. Hawker et al. (20) have shown that serum stimulation of quiescent rat and chick cells leads to a biphasic induction of AP-1 activity and Howe et al. (7) have implicated a rise in MAP kinase levels in the progression to S phase of epithelial cells that are released from a transforming growth factor β1-imposed late G1 block. In addition, several reports have shown that an immediate peak of growth factor-stimulated MAP kinase can be succeeded by elevated activity lasting up to 4 h (21, 22), with the sustained activity having different biological consequences (23). Indeed, significant MAP kinase activity persists throughout G1 in serum-stimulated hamster and Rat-1 cells (24, 25), but the major peaks we observed with v-Src (Fig. 4a) do not seem characteristic of serum stimulation. It has also been shown that cells transformed by v-Src have elevated MAP kinase activity (8, 10), but since these studies did not examine variations during the cell cycle, it is not known whether this elevation persists throughout the cell cycle. Finally, Tamemoto et al. (26) have observed that, as Chinese hamster ovary cells traverse the cell cycle, MAP kinases are activated around the M phase as well as at the start of G1.

None of these studies has systematically followed the behavior of AP-1 and MAP kinase as cells progress from Go to S phase under the impetus of a single mitogen nor, with the single exception of a study with metabolic inhibitors (25), have they examined the consequences of withdrawal and reinstatement of the mitogenic stimulus during G1. This facility has enabled us to show peaks of AP-1 DNA binding and MAP kinase activities that reflect the need for v-Src in
Fluctuations of MAP Kinase and AP-1 by v-Src

(a) MBP

(b) MAP kinase activity (cpm Cerenkov)

(c) Thymidine incorporation (cpm x 10^4)

Time after stimulation (hours)
leaving G₀, passing the G₁ pause point, and passing the commitment point for DNA synthesis (Figs. 1, 4, and 6; the MAP kinase peaks observed later in S phase could reflect culture asynchrony or the M-phase peak reported by Tamemoto et al. (26)). Moreover, pulses of v-Src show that G₁ elevations of MAP kinase activity require persistent v-Src functioning, and the loss of the peak immediately before S phase is accompanied by failure to initiate DNA synthesis (Fig. 4, b and c). The inhibitory effect of the ΔRaf-1:ER construct on v-Src induced DNA synthesis and MAP kinase activation (Fig. 6, a and b) also strongly suggests that the Raf-1/MAP kinase pathway is essential for v-Src mitogenesis. Moreover, although oncogenic Src is known to activate c-Raf-1 (18), and this probably occurs by the two proteins forming a functional complex (27), Fig. 7a represents the first evidence that v-Src exerts its mitogenic effect through physical association with Raf-1.

These novel findings indicate that v-Src, in common with certain growth factors (11, 22, 28, 29), requires an intact MAP kinase pathway for mitogenesis, although the pattern of MAP kinase response induced by serum mitogens (24, 25) and v-Src differs. It has also been reported that inhibition of the MAP kinase pathway reverts v-Src transformation (11), findings that apparently conflict with the data on transformation displayed in Fig. 7b. Possible explanations for this discrepancy include the different cell types studied and the fact that Cowley et al. (11) used microinjection of a dominant negative MAP kinase construct, which might have had more extreme effects than did ΔRaf-1:ER in R29RER-8. Clearly, if MAP kinase is needed for transformation, less is required than for initiation of DNA synthesis. It is pertinent that we have shown previously that AP-1 functioning is also needed for v-Src-induced mitogenesis, but not transformation, of chick cells (5).

We are not aware that any previous work has revealed fluctuations of MAP kinase activity throughout G₁ and their possible importance. The combination of conditional v-Src activation and inhibition of Raf-1 used in our study imply that MAP kinase activation is required for the transit of G₁ at stages in addition to the need at exit from G₁ described in studies on other mitogenic stimuli. The second peak of MAP kinase activity, when curtailed by removal of the v-Src stimulus, is nonetheless associated with a significant proportion of cells reaching a pause point (Fig. 4, b and c). Such cells do not display later peaks of MAP kinase activity and do not reach S phase (Fig. 4b). However, reinstatement of the v-Src stimulus leads to increases in both MAP kinase activity and subsequent DNA synthesis, suggesting that MAP kinase is also required for executing the v-Src-induced commitment to S phase (Fig. 4c). Meloche (25) has suggested that a drop in MAP kinase activity is a prerequisite for S-phase entry, and Fig. 4, a and c, taken in isolation, could indicate that the marked increase in MAP kinase activity from 20 h onwards in Fig. 4c, attenuates DNA synthesis. However, the totality of evidence that we present in Figs. 4 and 6 indicate that late G₁ increases in MAP kinase activity are important for S-phase entry, whether or not their subsequent diminution is required for completion of DNA synthesis. Final proof that MAP kinase is essential at any particular point in G₁ will, however, require the use of an inducible inhibitor of this pathway, in concert with ts v-Src, and we are currently trying to devise such an approach.

Specific, inducible inhibition of MAP kinase, when available, will answer another question raised by this study. The oscillations in MAP kinase and AP-1 DNA binding activities are parallel, if not simultaneous, and are closely coupled to v-Src activity. It is not clear, however, whether they are independent consequences of v-Src function or whether they are linked, and MAP kinase has a positive or negative effect on AP-1 DNA binding. A major positive effect of MAP kinase on AP-1 is through elevation of c-fos transcripts (reviewed in Ref. 30), but this is never seen during v-Src-
driven transit of G₁ (Fig. 2a), although it does occur if v-Src is activated in the presence of serum growth factors. MAP kinase can also phosphorylate COOH-terminal sites on c-Jun, with a negative effect on AP-1 DNA binding (31) and, although it has been reported to phosphorylate NH₂-terminal serine residues that positively regulate c-Jun transcriptional activity (32), more recent evidence attributes this function to the JNK serine/threonine kinases (33, 34). The evidence that AP-1 DNA binding is positively modulated posttranslationally by v-Src (Fig. 2b and c, and Fig. 3) is, therefore, difficult to explain in terms of MAP kinase activity, and we have not yet investigated the effect of v-Src on JNK activities. Thus, the mechanism of AP-1 activation in our cells remains moot, and inducible inhibition of the MAP kinase pathway should reliably establish whether its functioning reduces or enhances AP-1 DNA binding.

4 A. W. Wyke, unpublished data.

Given the likely importance of these oscillating activities during G₁, the question arises of the functions that they serve, which may differ at different points of cell cycle transit. The nature of the AP-1 complexes appears uniform throughout G₁ (Fig. 3), but this does not rule out subtle changes in their activity or in the spectrum of genes to which they gain access. The cellular homeostatic mechanisms that modulate fluctuations in activity may also vary. Thus, not all immediate early genes respond to v-Src in the pattern displayed by MAP kinase and AP-1. The MAP kinase-specific phosphatase, CL100, is encoded by an immediate early transcript (35) whose response to v-Src is typically transient,¹ a finding that also suggests that CL100 does not regulate the mid G₁ oscillations in MAP kinase activity. In conclusion, v-Src-induced transit of G₁ is typified by sequential elevations of MAP kinase and AP-1 activities that are likely to be important in mitogenesis. The periods during which these elevations occur may represent crucial phases in G₁, possibly the times at which pivotal changes occur in the determinants of cell cycle progression. We are, therefore, also investigating the effects of v-Src on the G₁ cyclins,
their cognate cyclin-dependent kinases, and their regulators and effectors.

Materials and Methods

Cell Culture. Rat-1 tsLA29 cells (36) were maintained in DMEM supplemented with 5% NBCS at 35°C (permissive temperature). To render cells quiescent, they were plated at a density of 6 × 10^4 cells/cm^2 and incubated at 39.5°C (restrictive temperature) for 24 h. The medium was then replaced with DMEM containing 0.25% NBCS, and the incubation continued for a further 48 h.

An estrogen-inducible, amino terminally truncated, oncogenic Raf-1 construct (hrAfER, now known as ΔRaf-1;ER; Refs. 15 and 17; kindly provided by Dr. M. McMahon, DNAX, Palo Alto, CA) was transfected into Rat-1 tsLA29 cells, and single-cell colonies were isolated at restrictive temperature. These cells were grown as described above, but after plating for quiescence, the medium was replaced with DMEM lacking phenol red and containing 0.3% charcoal-stripped NBCS.

DNA Synthesis. DNA synthesis was measured by pulse-labeling of the cells for 1 h in the presence of [methyl-3H]thymidine at a final concentration of 1 μCi ml^-1. The dishes were washed once in cold PBS, and DNA precipitated in situ by the addition of cold 5% trichloroacetic acid. After washing with methanol, samples were dissolved in 0.03 M NaOH prior to liquid scintillation counting.

MAP Kinase Assays. MAP kinase activity was measured in an in vitro assay after immune precipitation with an antiserum raised against the same COOH-terminal peptide region of p42 ERK2 MAP kinase as was used by Levers and Marshall (37). Cells from 60-mm dishes were lysed on ice in 1 ml of lysis buffer containing 20 mM Tris (pH 8.0), 40 mM sodium PPF, 50 mM NaF, 5 mM MgCl₂, 100 μM sodium orthovanadate, 10 mM EGTA, 6 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, and 40 μg/ml aprotinin. Cellular debris was removed by centrifugation prior to immune precipitation of 50 μg protein with antiserum conjugated to protein A-Sepharose beads. Immune complexes were washed three times in lysis buffer without MgCl₂, once in 30 mM Tris (pH 8.0), and finally in kinase buffer [30 mM Tris (pH 8.0), 20 mM MgCl₂, and 2 mM MnCl₂]. The beads were resuspended in 30 μl of kinase buffer containing 3 μM ATP, 0.5 μCi [γ-32P]ATP, and 7.5 μM MBP as the substrate and incubated at 30°C for 30 min. After resolution by SDS-PAGE in 12.5% gels, the radioactive products were transferred to Immobilon-P membrane and located by autoradiography. Quantitation was by excision of the radioactive MBP and Cerenkov counting.

Electrophoretic Mobility Gel Shift Assays. Cells from 100-mm dishes were lysed in 100 μl of buffer containing 20 mM HEPES (pH 7.9), 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 1 mM DTT, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, and a mixture of protease inhibitors consisting of pepstatin A, leupeptin, and aprotinin, all at 5 μg/ml, together with 1 mM benzamidine and 0.25 mM phenylmethylsulfonyl fluoride. DNA binding assays were performed in 20 mM HEPES buffer (pH 7.9) with 50 mM NaCl, 5 mM MgCl₂, and 3 μg poly dC-dG oligodT as the probe in a total volume of 20 μl, and after incubation on ice for 30 min, complexes were resolved by electrophoresis in 4% polyacrylamide gels. Nuclear extracts were prepared as described previously (2).

Oligonucleotides were synthesized in house using Applied Biosystems technology and end-labeled by incubation with [γ-32P]ATP and T4 polynucleotide kinase. The oligonucleotide probe used for the AP-1 DNA binding assay was the collagenase AP-1 site: AGCTTTGACCACTTCACTGCT (written from 5’ to 3’).

For the UV cross-linking experiments, this oligonucleotide was synthesized with bromodeoxyuridine instead of thymidine, thus conferring increased sensitivity to cross-linking by UV light. The DNA binding assays were performed on a 5-fold increased scale, and the electrophoresis was performed with one-fifth of the samples run as marker tracks and the remaining four-fifths run together. The wet gels were wrapped in Saran wrap and irradiated with UV light from a transilluminator for 30 min. The marker tracks were dried and exposed to X-ray film for autoradiography, while the portion of the gel containing the remaining cross-linked complexes was excised from the rest of the gel and soaked in SDS-PAGE sample buffer at 37°C for 15 min. This was then rotated 90° and laid on the stacking portion of a 10% SDS polyacrylamide gel for resolution in a second dimension. These DNA-protein complexes were then visualized after autoradiography. For antibody-protein interaction experiments, antibodies were incubated on ice with the protein extracts for 30 min prior to the addition of the oligonucleotide probe.

RNA and Protein Analysis. Total cellular RNA was prepared using RNeasy reagent (Qiagen, Ltd.), and Northern blot analysis was carried out using standard techniques (38). The c-fos probe used was the 1.3-kb BglII-PvuII restriction fragment of v-fos (39). Immunoprecipitation and Western blot analyses were carried out using standard techniques (38).

Antibodies. The antibodies used were all polyclonal rabbit sera raised against: (a) a segment of v-Fos spanning the basic region and conferring pan-Fos specificity (Ab 388-4; Ref. 40); (b) full-length bacterial c-Jun protein and specific to c-Jun (Ab 730-5; Ref. 41); and (c) a peptide from the COOH terminus of p42 ERK2 MAP kinase (Ab 945; Ref. 37). Commercial sera used were v-Src Ab-1 from Oncogene Science (purchased from Cambridge Bioscience) and anti-human estrogen receptor (F3) ER-F3-A from Euromedex (Souvereweyshire, France).

Acknowledgments

We are grateful to Martin McMahon and DNAX for providing the ΔRaf-1;ER construct, to Chris Marshall for communicating data prior to publication, and to Brad Ozanne for critical reading of the manuscript.

References


2. Welham, M. J., Wyke, J. A., Lang, A., and Wyke, A. W. Mitogenesis induced by pp60
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