Isolation of Temperature-sensitive Mutations in the c-raf-1 Catalytic Domain and Expression of Conditionally Active and Dominant-defective Forms of Raf-1 in Cultured Mammalian Cells

Kimberly K. Lu, Alex V. Bazarov, Luke S. Yoon, and John M. Sedivy

Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, Rhode Island 02912

Abstract

The c-Raf-1 kinase is converted into an oncprotein by functional inactivation of its NH2-terminal regulatory domain and into a dominant-interfering protein by mutations that eliminate catalytic activity. This report describes a systematic charged residue-to-alanine scanning mutagenesis of the ATP-binding subdomain of the c-raf-1 gene. Two temperature-sensitive mutations were found, which were then used to construct both conditionally active and conditionally dominant-defective alleles. Stable cell lines overexpressing both types of mutants were isolated, and their phenotypes were examined. Ectopic expression of Raf-1 activity in quiescent cells was not sufficient to elicit S-phase entry, but the Raf signal could be efficiently complemented by the progression factor insulin-like growth factor I. The results point to a function of Raf-1 in the platelet-derived growth factor and epidermal growth factor pathways, leading to the establishment of competence for cell cycle entry. Ectopic expression of the dominant-defective activity in quiescent cells efficiently blocked entry into S phase. Effects of the dominant-defective protein could be detected minutes after the shift to the restrictive conditions and resulted in the rapid down-regulation of the mitogen-activated protein kinase pathway. Taken together, the phenotypes of the conditionally active and conditionally dominant-defective mutants point to a critical function of Raf-1 at very early times during exit from G0 and entry into G1.

Introduction

The c-raf-1 gene encodes a cytoplasmic serine/threonine protein kinase, the activity of which has been implicated in the regulation of proliferation and differentiation (reviewed in Refs. 1–3). Raf-1 protein can be activated by many signals, including growth factors, differentiation hormones, tumor promoters, inflammatory cytokines, calcium mobilization, DNA-damaging agents, and oxygen radicals. Raf-1 acts in a signal transduction pathway from growth factor receptors to the nuclear immediate early response, which involves Ras, Raf-1, and the MAPK5 cascade (reviewed in Refs. 4 and 5). Ras-mediated translocation of Raf-1 to the plasma membrane is a crucial step in the activation process (6–8).

The c-raf-1 proto-oncogene is converted into a dominantly acting oncogene by mutation (such as truncation) of the NH2-terminal regulatory domain (9, 10). Conversely, mutations that eliminate kinase activity display a dominant-defective phenotype (11). The role of Raf-1 as a key inducer of the MAPK pathway is indicative of a function early in the G0-S transition. Whether Raf-1 performs functions at other stages of the cell cycle has not been examined, but several indirect lines of evidence suggest that this may be the case (12–14). Conditional mutants would be ideal tools to analyze the requirement for Raf-1 during cell cycle progression. Toward this end, we sought to isolate ts alleles in the c-raf-1 kinase domain.

ts mutations have been isolated in several protein kinase genes. The largest number have been obtained by in vivo selections or screens, which are possible only in genetic systems that allow the analysis of numerous candidates. This collection includes mutations in Saccharomyces cerevisiae CDC28 (15), casein kinase I (16), casein kinase II (17), and CDC7 (18); Schizosaccharomyces pombe Cdc2 (19); Aspergillus nidulans NIMA (20); and Drosophila melanogaster sevenless protein kinase (21) and D-ras (22). Among higher eukaryotic kinases, ts mutations in several retroviral oncopogenes have been identified: v-src (reviewed in Ref. 23), v-erbB (24), v-fps (25), v-mos (26), and v-ros (27). Only one ts mutation in a mammalian cellular kinase gene, cdc2 in the murine FT210 cell line, has been reported (28).

In several instances, ts mutations were obtained by site-directed mutagenesis of residues corresponding to known ts

Received 10/6/97; revised 3/13/98; accepted 4/9/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by NIH Grant GM-R01-55435 and Presidential Young Investigator Award DMB-9057715 from the National Science Foundation (to J. M. S.).

2 The first two authors contributed equally to this work.

3 Present address: M.D. Program, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115.

4 To whom requests for reprints should be addressed. Phone: (401) 863-7691; Fax: (401) 863-1291; E-mail: john_sedivy@brown.edu.

---

The abbreviations used are: MAPK, mitogen-activated protein kinase; ts, temperature-sensitive; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PDGF, platelet-derived growth factor; FACS, fluorescence-activated cell sorting; EGF, epidermal growth factor; IGFI, insulin-like growth factor I; CS, calf serum; PKA, cAMP-dependent protein kinase; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; GAT, chloramphenicol acetyltransferase.
Lesions in related kinase genes. For example, both v-abl (29, 30) and v-ck (31) mutations were successfully modeled on v-src mutations, and a Drosophila MEK mutation was based on a known S. pombe cdc2 mutation (32). This approach is limited by two constraints. First, with the exception of a few highly conserved residues involved in catalysis, the sequence conservation between kinases is low (33). Therefore, mutagenesis by modeling is likely to be successful only with closely related kinases. Second, the temperature window of conditionality is dependent on the organism in question; for example, only a small fraction of v-src mutations, originally isolated in chicken cells at 41.5°C, were ts when incorporated into c-src of murine cells, which must be grown at lower temperatures (34, 35).

The third approach to isolating ts mutations has been in vitro scanning using either small in-frame insertions or deletions. This method was successfully used with v-abl (36), v-src (37), and S. cerevisiae CDC7 (18). However, the yield of ts mutations among total mutations is low (37). Another method of in vitro scanning, designated “alanine scanning,” involves replacing clusters of charged residues with alanine (38, 39). This approach has been used to isolate ts mutations with relatively high frequency (40). This report describes an alanine scanning mutagenesis of the ATP-binding subdomain of the c-raf-1 gene. This approach was chosen instead of incorporating the one known D-raf ts mutation (22) into the mammalian gene because the nonpermissive temperature for the Drosophila mutant is only 20°C. Two ts mutations were found, which were then used to construct both conditionally active and conditionally dominant-defective alleles of the c-raf-1 gene. The ability to turn Raf activity both on and off using simple temperature shifts is a valuable tool for investigating the requirement for c-Raf-1 at various stages of the cell cycle.

Results
Isolation of ts Mutations in the Kinase Domain of the c-raf-1 Gene. NH2-terminally deleted c-Raf-1 derivatives act as activated oncoproteins: they display deregulated kinase activity and transform rodent fibroblast cell lines. Stanton et al. (9) reported that a deletion mutant lacking 305 NH2-terminal residues (22W) had maximum kinase activity and that larger deletions (313 and 324 residues) displayed progressively reduced kinase activity. However, even the 22W mutant had an activity that was 5-fold lower than that of v-raf. In contrast, Heidecker et al. (10) reported a mutant deleted for 334 NH2-terminal residues (EC12), which displayed kinase activity that was comparable to that of v-raf. EC12 was chosen as the starting point for the work reported here.

Pairwise alanine-scanning mutations were introduced into the entire ATP-binding subdomain of EC12 (Fig. 1). Following site-directed mutagenesis and verification by DNA sequenc-
ing, plasmid DNAs were electroporated into the rat fibroblast cell line TGR-1 to ascertain the biological activity of the mutant Raf-1 proteins by parallel assays of focus formation (data not shown) and growth in soft agar (Table 1). Assays were performed in triplicate at three temperatures: 31.5°C, 37°C, and 39.5°C. The majority of the mutants (7 of 12) were defective at all three temperatures. Three mutants were not distinguishable from the unmutagenized control (EC12), and two mutants (K378/D381 and R391/E393) were ts (Fig. 1). Consistent results were obtained in at least three independent experiments, and the focus formation and soft agar growth assays were always in agreement. The mutants K378/D381 and R391/E393 were chosen for further study and were designated EC12-ts1 and EC12-ts2, respectively.

Individual colonies of mutants EC12-ts1 and EC12-ts2 were picked from soft agar plates that were incubated at 31.5°C and expanded into clonal cell lines. Several independent cell lines of each mutant were tested for plating efficiency in soft agar at 31.5°C, 37°C, and 39.5°C. The results demonstrated a clear temperature dependence of anchorage-independent growth (Table 2). Microscopic observation showed that cells grown at 31.5°C displayed a transformed morphology, and cells grown at 39.5°C displayed a nontransformed morphology (Fig. 2). Cellular morphology was reversible in either direction upon temperature shift. Due to the slow growth of TGR-1 and NIH-3T3 cells at 31.5°C, following the initial characterization of the mutants, the permissive temperature was raised to 34°C to facilitate additional experiments. The behaviors of both EC12-ts1 and EC12-ts2 mutants are identical at 31.5°C and 34°C (data not shown).

### Table 1 Soft agar colony assays following electroporation of mutant DNA plasmids

The values presented are numbers of colonies per plate on equivalent dilutions of the electroporation mixture (1:20). Each value is an average of two plates. Aliquots of all electroporations were also plated for G418-resistant colony formation at all temperatures (the EC12 expression plasmid contains a neo marker). The electroporation efficiencies were equivalent among all the samples shown (data not shown). Mutants E345/E345, E345/E348, and K414/D415 displayed microcolonies that were visible by microscopic observation at 31.5°C. The plating efficiency of the wild-type (EC12) control was reproducibly 3- to 5-fold lower at 39.5°C than it was at 37°C. Consistent results were obtained in at least three independent experiments. Comparable results were obtained upon electroporation of NIH-3T3 cells (data not shown).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No. of colonies/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31.5°C</td>
</tr>
<tr>
<td>EC12 (wild type)</td>
<td>93</td>
</tr>
<tr>
<td>E345/E345</td>
<td>0</td>
</tr>
<tr>
<td>E345/E348</td>
<td>0</td>
</tr>
<tr>
<td>K365/K367</td>
<td>0</td>
</tr>
<tr>
<td>K367/H369</td>
<td>0</td>
</tr>
<tr>
<td>H369/D371</td>
<td>0</td>
</tr>
<tr>
<td>K378/D381 (EC12-ts1)</td>
<td>145</td>
</tr>
<tr>
<td>D381/E385</td>
<td>110</td>
</tr>
<tr>
<td>R391/E393 (EC12-ts2)</td>
<td>35</td>
</tr>
<tr>
<td>R398/K399</td>
<td>83</td>
</tr>
<tr>
<td>K399/R401</td>
<td>133</td>
</tr>
<tr>
<td>R401/H402</td>
<td>0</td>
</tr>
<tr>
<td>K414/D415</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 2 Efficiency of plating in soft agar of clonal cell lines transformed by Raf-EC12 mutants

Cells were trypsinized and counted, and 1000 cells were plated per 6-cm dish. Plates were incubated at 31.5°C, 37°C, and 39.5°C for 3 weeks, and macroscopic colonies were counted on photographic enlargements. The values presented are numbers of colonies per plate; each value is an average of two plates.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Cell line</th>
<th>No. of colonies/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31.5°C</td>
<td>37°C</td>
</tr>
<tr>
<td>EC12 (wild type)</td>
<td>A1-S</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>A2-S</td>
<td>NC*</td>
</tr>
<tr>
<td>D381/E385</td>
<td>D1-S</td>
<td>108</td>
</tr>
<tr>
<td>K378/D381</td>
<td>C1-S</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>C3-M</td>
<td>50</td>
</tr>
<tr>
<td>R391/E393</td>
<td>C6-L</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>E3-M</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>E4-M</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>E6-L</td>
<td>167</td>
</tr>
</tbody>
</table>

*NC, not counted.

**Characterization of Mutant Raf Kinase Proteins.** Expression of the mutant Raf proteins at permissive and restrictive temperatures was examined by immunoblot analysis. In cells transformed with the EC12 control plasmid, the M.36,000 Raf-EC12 protein was significantly overproduced, relative to the M.73,000 endogenous c-Raf-1 protein (Fig. 3A). Relative to the EC12 protein, the EC12-ts1 protein was expressed at essentially the same level, whereas the EC12-ts2 protein was overproduced 3-4-fold. All proteins were slightly underrepresented at the restrictive temperature, the effect being most pronounced with the EC12-ts1 protein (2-fold). The results presented here are representative of a panel of nine cell lines, three each of EC12, EC12-ts1, and EC12-ts2 (data not shown).

In vivo activity of the mutant Raf-EC12 proteins was examined by measuring the activation of an AP-1-dependent reporter gene in transient transfection assays. NIH-3T3 cells were coelectroporated with the CAT reporter plasmid pB4X (41) and the EC12 constructs encoding wild-type or mutant Raf-1 kinase domains. Each electroporated sample was split into two equal aliquots, which were subsequently incubated at permissive and restrictive temperatures (Fig. 3B). EC12 stimulated AP-1 activity 8-10-fold. The magnitude of the stimulation relative to the empty vector control was the same at both temperatures, but the specific activities (units activity per mg protein) were 2-3-fold lower at the restrictive temperature. This was equally true of the EC12 and the control (empty vector) transfections. Although a natural thermosensitivity of AP-1 activity cannot be ruled out, a contributing factor was faster cell growth at the higher temperature, which had the effect of diluting productively transfected cells.

The EC12-ts2 mutant activated AP-1 in a thermosensitive fashion: the ratio of activities at the permissive and restrictive temperatures was 8.8-fold, compared to 2.8-fold with the wild-type EC12 control. The EC12-ts1 mutant was not obviously thermosensitive in this assay (permissive restrictive temperature ratio of activities was 3.2). Neither mutant displayed entirely wild-type levels of activity at the permissive temperature. Both mutants were also somewhat leaky at the
restrictive temperature: in comparison to the empty vector control, EC12-ts1 and EC12-ts2 elicited 4.5- and 2.5-fold elevated AP-1 activities, respectively.

Activation of the MAPK pathway by temperature down-shift was examined in a TGR-1 cell line that was stably transfected with the EC12-ts2 protein (Fig. 2). Cells were made quiescent by serum deprivation at the restrictive temperature, cultures were shifted to the permissive temperature, and samples were collected at successive time points, displayed by SDS-PAGE, and immunoblotted with an antibody that was specific for the phosphorylated forms of ERK1 and ERK2 (Fig. 3C). As a control, either serum or PDGF was added at the time of down-shift. The results showed that activation of the EC12-ts2 protein by temperature down-shift alone resulted in a robust activation of the endogenous ERK1 and ERK2 proteins. Comparable results were observed when
the immunoblots were reprobed with an antibody specific for the phosphorylated form of MEK (data not shown). When later time points in an analogous experiment were immunoblotted with antibody to cyclin D1, no induction was observed upon temperature down-shift in the absence of serum or PDGF (data not shown).

In vitro kinase activity of mutant Raf-EC12 proteins was examined by measuring the phosphorylation of a recombinant kinase-inactive MEK substrate protein in immunoprecipitation kinase assays. Raf proteins were immunoprecipitated from clonal cell lines expressing EC12, EC12-ts1, and EC12-ts2 proteins, and kinase assays were performed at 23°C (Fig. 4A) and 30°C (Fig. 4B). EC12 and EC12-ts1 proteins displayed similar levels of activity both at 23°C and 30°C. The EC12-ts2 protein was significantly defective at both temperatures, with some degree of temperature dependence: relative to EC12, activity was 5.8-fold lower at 23°C and 13.5-fold lower at 30°C. Using the criterion of in vitro kinase assays, the ts2 mutant is clearly more impaired than the ts1 mutant. These data are consistent with the in vivo phenotypes elicited by the mutant EC12 proteins: cells transformed with EC12-ts2 display a tighter ts phenotype (Table 2), and higher expression levels of EC12-ts2 protein are required to elicit transformation (Fig. 3A).

**Induction of S-phase Entry by the Activation of Raf Kinase in Quiescent Cells.** Clonal cell lines expressing Raf-EC12 mutant proteins ts1 or ts2 were serum deprived for 48 h at the restrictive temperature. Quiescence was verified by FACS analysis, which showed that >90% of cells had a diploid DNA content. Quiescent cells were shifted to the permissive temperature without a change of medium, and at successive time points, samples were labeled with [3H]thymidine to monitor entry into S phase (Fig. 5). The results showed that the temperature down-shift was not sufficient to elicit S-phase entry. A total of six independent cell lines (three each of ts1 and ts2) were examined on at least three separate occasions with consistent results. As a control, cultures were stimulated with 10% serum at the time of down-shift. These cultures entered S phase in parallel with the TGR-1 control, showing that cells transformed with conditional Raf mutants are not intrinsically abnormal in the experimental regimen used. Analogous results were obtained at the restrictive temperature: all cell lines failed to enter S phase without serum stimulation, whereas all entered normally following serum stimulation (data not shown).

Samples collected in parallel during the [3H]thymidine uptake experiment were analyzed by immunoblotting for the presence of the mutant Raf-EC12 proteins (Fig. 6A). The EC12-ts2 protein was 2-fold underrepresented at the 0-h time point relative to the 18-, 22-, 26-, and 30-h time points. The same result was obtained with a cell line transformed with the ts1 mutant (data not shown). The reduced levels of Raf-EC12-ts2 protein found at the zero time point are probably due to the slight instability of the mutant Raf proteins at the restrictive temperature (Fig. 3A). Two observations indicate, however, that the failure of the cells to enter S phase is not due to the 2-fold lower expression level of mutant Raf-EC12 proteins in quiescent cells: first, in a separate experiment, samples collected at 2 h after down-shift showed the elevated expression characteristic of the permissive temperature; and second, extended incubation for up to 48 h did not elicit S-phase entry (data not shown). As a further control, the same batch of cells that was used for the [3H]thymidine uptake (Fig. 5) and immunoblotting (Fig. 6A) analyses was plated in soft agar (Fig. 6B). The culture displayed the expected thermosensitive transformed phenotype; this rules out the possibility that the culture used in this experiment had reverted (lost EC12-ts2 expression). It is, therefore, evident that Raf-1 kinase activity that is sufficient to cause transformation is not sufficient to cause quiescent cells to reenter the cell cycle.

We next examined the effects of activating Raf kinase in quiescent cells in combination with stimulation by individual growth factors. The tighter of the two Raf-EC12 mutants, ts2, was used in these experiments. The experimental regimen was to render cells quiescent by serum deprivation at the restrictive temperature, followed by a simultaneous temperature down-shift and addition of growth factors. [3H]Thymidine uptake was used to monitor entry into S phase. Control experiments with parental TGR-1 cells showed that low concentrations of PDGF (2 ng/ml; Fig. 7A, column 1), EGF (0.1 ng/ml; Fig. 7A, column 4), or IGF-I (10 ng/ml; Fig. 7A, column 7) added singly did not elicit S-phase entry. However, when
Temperature-sensitive Mutants of Raf-1 Kinase

Fig. 5. Absence of S-phase entry following the activation of Raf-EC12 proteins in quiescent cells. Cells were made quiescent at the restrictive temperature. At the zero \( t = 0 \) h time point, all cultures were shifted down to the permissive temperature. The indicated samples \( (\Delta, \Phi, \Psi, \Theta) \) were serum stimulated at \( t = 0 \) h; the remainder \( (\triangle, \sqcap, \triangledown, \bigcirc) \) continued to be incubated in 0.25% CS. At the indicated times, \( ^{3}H \)thymidine was added, and incubation was continued for 1 h, at which time the sample was processed for TCA-precipitable \( ^{3}H \)cpm. Data points, averages of two independent determinations; values for each cell line were normalized to the peak value (set to 100%) to correct for slight differences in cell density for each culture. The raw peak cpm values were within a range of 20% (data not shown). Pink-1 and ts-1 (A) and ts-2 and ts-2 (B) are independent, TGR-1-derived, clonal cell lines transformed with the EC12-ts1 and EC12-ts2 mutants, respectively. Data from a single representative experiment are shown. The results presented were reproducible in three independent trials.

these growth factor treatments were combined, efficient entry into S phase was observed (Fig. 7A, column 14). Supra-
physiological concentrations of either PDGF (Fig. 7A, columns 2 and 3) or EGF (Fig. 7A, columns 5 and 6) acting alone could trigger significant entry into S phase, whereas even very high concentrations of IGF-I (100 ng/ml) were without effect (Fig. 7A, columns 8 and 9). These results are in excel-
lent agreement with the original literature describing the G0\( \rightarrow \)S progression in the BALB/c-3T3 model system (re-
viewed in Ref. 42).

Activation of the EC12-ts2 protein, combined with low concentrations of PDGF (Fig. 7B, columns 3 and 4) or EGF (Fig. 7B, columns 5 and 6), elicited a small (~2-fold) enhancement of S-phase entry, relative to growth factor treat-
ment alone. Because both PDGF and EGF are known to strongly stimulate c-Raf-1 in NIH-3T3 cells, c-Raf-1 is con-
sidered to be a downstream component in these signaling pathways (3, 5). Consistent with this view, the data presented here indicate that activation of a ts Raf kinase protein can enhance the effects of limiting amounts of PDGF and/or EGF by augmenting downstream signaling. However, the magni-
tude of downstream signaling elicited by the ectopic activation of the conditional EC12-ts2 protein is weak, which is also consistent with the observation that this signal alone is a poor mitogen.

In contrast, the combination of Raf kinase activation and IGF-I stimulation resulted in strong S-phase entry (Fig. 7B, column 8). The enhancement was 6.5-fold relative to IGF-I treatment alone and 7.9-fold relative to Raf activation alone.

To confirm these results, S-phase entry was monitored in a separate experiment for an extended period of time (Fig. 7C). The S-phase entry elicited by Raf kinase and IGF-I and by the combined effects of PDGF, EGF, and IGF displayed identical kinetics, with a peak at 26 h poststimulation. The peak Raf/ IGF value was 38.3% of the PDGF/EGF/IGF value and 11.7-
fold greater than the value elicited by Raf activation alone. Samples collected in parallel and subjected to FACS analysis corroborated these results (data not shown). We conclude that Raf kinase activation generates a downstream signal in the PDGF and/or EGF pathways that is insufficient to cause S-phase entry because it lacks an essential second signaling component that can be provided by the IGF-I pathway.

Construction of a Conditional Dominant-defective Mu-
tant of c-Raf-1. A kinase-inactive mutation in full-length c-raf-1 is known to confer a strong dominant-defective pheno-
type (11). A ts mutation affecting kinase activity should, therefore, confer a conditional dominant-defective pheno-
type. To test this hypothesis, we constructed the R391/E939 (ts2) mutation in the full-length human c-raf-1 cDNA. The cDNA was expressed in the retrovirus vector pMNC (11), and the mutation was verified by sequencing (data not shown). Plasmid DNA was electroporated into \( \Psi \)2 packaging cells (43), and viral supernatants were used to infect TGR-1 and NIH-3T3 cells. Selection was at the permissive temperature using 10 mg/ml G418 to enrich for clones with high expres-
sion levels (44).

Individual clones were expanded into cell lines, and Raf-1 expression was assayed by immunoblotting (it should be
Figure 6. Expression of EC12-ts2 protein in quiescent cells before and after down-shift to the permissive temperature. A, immunoblot analysis of ts2-2 samples collected in the experiment shown in Figs. 5, B, soft agar colony assay of the ts2-2 culture used in this experiment. The immunoblot was probed with the anti-Raf antibody PBB1. Equal amounts of total protein were loaded in each lane (see "Materials and Methods"). Left, migration position of the EC12-ts2 protein. Densitometric analysis showed that the relative band intensities for the 0- and 18-h time points were 1.00 and 2.09, respectively. In the exposure shown, endogenous c-Raf-1 is below the detection limit. CB (cross-reacting band), a M, 80,000 protein of unknown identity detected by the PBB1 monoclonal antibody. Right, migration positions (in kDa) of molecular weight standards (Bio-Rad, low molecular weight prestained). Data from a single representative experiment are shown. The results presented were reproducible in two independent trials. Analysis of the ts1-2 cell line produced identical results (data not shown).

Noted that the endogenous c-Raf-1 and the introduced Raf-ts2 proteins are of identical mobility and, therefore, cannot be differentiated by immunoblotting. Among eight TGR-1 and six NIH-3T3 derivatives analyzed, only two NIH-3T3-derived cell lines (designated Rif5 and Rif8) showed detectable levels of Raf-1 overexpression (data not shown). Both cell lines overexpressed Raf-1 protein at restrictive as well as permissive temperatures (Fig. 8A). The Rif8 cell line reproducibly showed a somewhat higher level of overexpression, especially at the restrictive temperature. As was the case with the EC12-ts1 and EC12-ts2 proteins, the Raf-ts2 protein accumulated to lower levels at the restrictive temperature. Interestingly, although the underphosphorylated (faster migrating) form of the Raf-ts2 protein accumulated at the permissive temperature, the phosphorylated (slower migrating) form accumulated at the restrictive temperature. The reasons for this phenomenon are currently not understood.

To determine the effect of the dominant-defective Raf-ts2 protein on the major known Raf target, the MAPK pathway, samples were collected at short intervals following serum stimulation at the restrictive temperature and immunoblotted with a monoclonal antibody specific for the active, phosphorylated forms of ERK1 and ERK2 (Fig. 8B). As expected, no signal was observed in quiescent cells, although the presence of the ERK1 and ERK2 proteins was evident by probing with an antibody directed against a nonphosphopeptide epitope (data not shown). Phosphorylation of ERK1 and ERK2 was strongly evident 1 min after serum stimulation and persisted for over 30 min in NIH-3T3 cells. In contrast, in Rif8 cells, both ERK1 and ERK2 phosphorylation was significantly reduced by 10 min following serum stimulation, and remained low at the 30-min time point. The results show that the dominant-defective Raf-ts2 protein did not abrogate the initial stimulation of the MAPK pathway by serum but that activity of the pathway decayed rapidly and remained at a low level.

Growth Properties of Cells Expressing the Dominant-defective Raf-ts2 Protein. To establish the most stringent restrictive condition possible, the growth of NIH-3T3 cells was quantitatively examined at temperature increments of 0.5°C, from 39°C to 41°C. Growth rate was relatively constant up to (and including) 40.5°C, after which point a precipitous drop in growth occurred (Fig. 9A). It was concluded that 40.5°C is the maximum temperature at which this cell line can be grown without gross temperature effects, and 40.5°C was adopted as the restrictive temperature for experiments with the Raf-ts2 protein.

The growth properties of the Rif5 and Rif8 cell lines were examined in a large number of independent experiments and can be summarized as follows. First, both Rif5 and Rif8 cell lines always grew significantly slower at the restrictive temperature than at the permissive temperature and also grew slower than the NIH-3T3 parental cell line at the restrictive temperature. Second, the Rif8 cell line always grew slower than the Rif5 cell line (this observation is in agreement with the relative expression levels of the Raf-ts2 protein in the two cell lines). Third, under no condition was growth abolished completely at the restrictive temperature. Fourth, growth at the permissive temperature was somewhat variable; in some experiments, no effect was observed (Fig. 9A), whereas in others, a slight growth defect was apparent (Fig. 9, B and C). Growth was influenced by factors such as previous passage history and initial cell density, and it is the only parameter in which the behavior of the Rif5 and Rif8 cell lines was found to vary from experiment to experiment. The most probable explanation is that the Raf-ts2 protein exhibits some growth-suppressive properties, even at the permissive temperature. This is consistent with the observations that the kinase activity of the EC12-ts2 protein was impaired at the permissive temperature (Figs. 3B and 4) and that Raf-ts2-expressing cell lines were difficult to isolate.

Abrogation of S-phase Entry by the Dominant-defective Raf-ts2 Protein. The cell lines Rif5 and Rif8 were serum deprived for 48 h at the permissive temperature. Quiescence was verified by FACS analysis, which showed that ~90% of cells had a diploid DNA content. Quiescent cells were stimulated with prewarmed (40.5°C) fresh medium containing 10% CS and immediately placed in a 40.5°C incubator. At successive time points, samples were harvested by trypsinization, stained with propidium iodide, and analyzed by flow cytometry (Fig. 10, A-D). The results showed that both the Rif5 and Rif8 cell lines were defective in exit from G1/S. The defect was most pronounced in Rif8 cells, in which the lowest G1/S phase value was 73%, as compared to 34% for NIH-3T3 cells. In agreement, the highest S-phase value was 20% for Rif8 cells, as compared to 63% for NIH-
Fig. 7. Requirement for IGF-I during Raf-induced entry into S phase. TGR-1 and ts2-2 cells (Figs. 5 and 6) were made quiescent at the restrictive temperature. At the zero (t = 0 h) time point, cells were shifted down to the permissive temperature and treated with the indicated concentrations of growth factors. PDGF, 1× 2 ng/ml; 3× 6 ng/ml; and 10× 20 ng/ml. EGF, 1× 0.1 ng/ml; 3× 0.3 ng/ml; and 10× 1 ng/ml. IGF-I, 1× 10 ng/ml; 3× 30 ng/ml; and 10× 100 ng/ml. The efficiency of S-phase entry triggered by 10% serum (determined by in situ bromodeoxyuridine incorporation) was 95–100% (data not shown). Data points, averages of two independent determinations; numbers above columns, actual values of individual data points (%). Data from individual representative experiments are shown; results were reproducible in at least two independent trials. A, response of TGR-1 cells to PDGF, EGF, and IGF-I. Thymidine was added at t = 24 h, and incubation was continued for 1 h. Values of 3H incorporation were normalized to the sample treated with PDGF/EGF/IGF (column 14). The PDGF/EGF/IGF value was 37% of the control stimulated with 10% serum. B, response of ts2-2 cells to PDGF, EGF, and IGF-I. Experiment was performed as in A, and data were normalized to the PDGF/EGF/IGF samples (data not shown). The PDGF/EGF/IGF value for the ts2-2 cell line was 33% of the 10% CS control. C, time course of IGF-I-stimulated S-phase entry of ts2-2 cells. Thymidine was added at the indicated time points, and incubation was continued for 1 h. Data were normalized to the PDGF/EGF/IGF peak value. In this experiment, the PDGF/EGF/IGF peak value was 63% of the 10% CS control.

3T3 cells. The values for Rf5 cells were intermediate between those of Rf8 and NIH-3T3 cells. A defect was also observed at the permissive temperature but was of significantly smaller magnitude. These observations are consistent with the growth properties of the cell lines.

S-phase entry was also examined using a single defined stimulus, recombinant PDGF. The cell lines NIH 3T3 and Rf8 were serum deprived for 48 h, PDGF was added directly to the medium at the indicated concentrations, and the cultures were immediately shifted to 40.5°C (Fig. 10, E and F). Entry into S phase was monitored by labeling with tritiated thymidine. The results showed that, whereas PDGF elicited robust S-phase entry in normal cells, this effect was significantly but incompletely inhibited by the Ts2-Raf-1 protein at the restrictive temperature. The magnitude of inhibition was comparable to that observed following stimulation with whole serum (Fig. 10, A–D). Therefore, the incomplete inhibition is likely due to an intrinsic leakiness of the Ts2-Raf-1 mutant rather than the complex mixture of mitogens present in whole serum.

Discussion

Construction of ts Mutations. The entire ATP-binding subdomain of the c-raf-1 gene was systematically scanned with pairwise charged residue-to-alanine replacements. All 12 possible mutant proteins have been tested, and 2 are ts. This is the first report of ts mutations in a mammalian Raf gene. Furthermore, it contributes to the growing list of reports in which alanine-scanning mutagenesis was used as an effective method for the isolation of ts mutations (40, 45, 46).

The choice of the ATP-binding domain as the target of mutagenesis was based on X-ray crystallographic studies of PKA (47), Cdk2 kinase (48), MAPK (49), and twitchin kinase (50). These studies indicate that the ATP-binding domain is a small, compact, and conserved structure and that the ATP-binding and peptide-binding domains are largely independent structural entities. Therefore, it is unlikely that mutations affecting the ATP-binding domain would affect the conformation and, thus, the substrate specificity of the peptide-binding domain. This hypothesis is supported by extensive mutagenesis studies of yeast PKA, which showed that mutants affected in ATP binding displayed an increased Kₘ for ATP but were not affected in the Kₘ for the peptide substrate (39). Furthermore, because of the structural conservation of the ATP-binding domain, mutations may be generalized to other protein kinases.

The five NH₂-terminal mutations were inactive in a biological transformation assay. This large cluster of null mutations spans the NH₂-terminal one-third of the ATP-binding domain, a region that contains the conserved glycine-rich loop.
which forms the nucleotide-binding fold. This region is highly conserved in the kinase structures solved to date (51, 52), and our mutagenesis data indicate that it is highly sensitive to perturbation. The conserved lysine (K375) adjacent to the glycine-rich loop was not mutated because a large body of evidence indicates that it is absolutely required for activity (53). Interestingly, the mutation immediately COOH-terminal to K375 (K375/D381; ts1) was ts. K375 coordinates the α- and β-phosphates of the ATP, and it is, thus, not surprising that structural perturbations in its vicinity would impair this function. Because the kinase activity of the Raf-EC12-ts1 mutant was not impaired at the restrictive temperature in vitro, the ts phenotype may be due to destabilization and/or increased turnover of the protein in vivo. Because this mutant was leaky at the restrictive condition, it would be reasonable to introduce additional mutations in this region to increase the tightness of the ts phenotype (45, 46).

The surprising result was that the second ts mutation (R391/E393; ts2) affected a glutamic acid residue (E393) that is absolutely conserved in all protein kinases (33). A mutation in the corresponding residue of the yeast PKA reduced enzymatic activity some 1000-fold (39); temperature sensitivity of the remaining activity was not tested. In comparison, the Raf-EC12-ts2 mutant at the restrictive condition was some 10-fold less active than the wild-type protein both in vivo (activation of an AP-1 reporter in transient transfection) and in vitro (immunoprecipitation kinase assay). It is, therefore, evident that whereas E393 is clearly important for activity, it is of relatively lesser importance than K375. This is consistent with structural data which indicate that, in several kinases, E393 is at some distance from the ATP-binding cleft (51, 52). E393 is, therefore, probably not involved in the initial binding of ATP to the protein but becomes reoriented during the activation process and participates in the ensuing catalysis. In summary, the data reported here indicate that E393 and the region immediately downstream of K375 are two rational targets for the isolation of ts mutations in protein kinases.

**Conditional Active Phenotypes.** The examination of cell lines stably expressing conditionally active Raf proteins revealed that ectopic expression of Raf activity in quiescent cells is not sufficient to cause progression into S phase. The failure to induce S-phase entry was not due to insufficient levels of Raf activity; in fact, the great majority of total Raf activity detected in extracts of EC12-transformed cells was contributed by the EC12 protein (data not shown). The cell lines were initially selected for growth in soft agar, which always resulted in the recovery of clones with high EC12 expression levels. This indicates that significantly higher levels of Raf activity are required for the maintenance of the transformed phenotype than for the growth of normal cells. Consistent with this hypothesis, clones selected for transformation with the EC12-ts2 mutant, which has impaired activity even at the permissive temperature, invariably displayed higher levels of expression.

The growth-promoting phenotypes of Raf were examined in more detail by investigating the interaction of Raf activity with the effects of individual growth factors. The G0→S transition has been subdivided into several temporal stages on the basis of differential growth factor requirements (42). Experiments with murine 3T3 fibroblasts showed that three growth factors, PDGF, EGF, and IGF-I, are necessary and sufficient to elicit a G0→S transition. We have previously shown that TGR-1 fibroblasts can be propagated in serum-free medium supplemented with these growth factors (54). Although PDGF and EGF are required at early times during the G0→S transition, IGF-I is required at later times to allow progression through the restriction point (42). Ectopic activation of Raf-EC12 proteins was found to augment the S phase-promoting effects of low concentrations of PDGF and EGF. This is consistent with a number of reports indicating that Raf is a downstream effector in both the PDGF and EGF pathways (3). Raf was found to cooperate strongly and synergistically with IGF-I to elicit S-phase entry. Two lines of evidence indicate that Raf and IGF-I function in distinct pathways. First, in contrast to PDGF and EGF, IGF-I is not a mitogen for rodent fibroblasts, even at very high concentrations. Second, stimulation with high concentrations of IGF-I does not activate endogenous c-Raf-1 in TGR-1 fibroblasts (data not shown). The finding that Raf cooperates with the progression factor IGF-I is consistent with the report that activated Ras cooperates with platelet-poor plasma to elicit S-phase entry (55).
The effects of ectopic Raf kinase activity in quiescent cells has been examined using a chimeric Raf-estrogen receptor protein, the kinase activity of which can be regulated with estrogen (56–58). Samuels et al. (56) reported an absence of S-phase entry, whereas Kerkhoff and Rapp (58) reported that Raf activation could elicit transition into S phase. The S-phase entry elicited by Raf (58) was not as robust as that elicited by serum, and it was not sufficient for continuous growth. Neither report examined the effects of progression factors such as plasma or IGF-I. Furthermore, because the ER moiety was fused in both cases to the COOH terminus of the Raf kinase domain, where the peptide-binding subdomain is located, the possibility that the fusion proteins may display altered substrate specificity has to be considered. Our results are in agreement with those of Samuels et al. (56).

**Conditional Dominant-defective Phenotypes.** The tighter of the two ts mutations (R391/E393; ts2) was incorporated into the full-length human c-raf-1 cDNA. A conditional dominant-defective gene has the obvious advantage in that it can be stably incorporated into permanent cell lines. Previous efforts to construct stable cell lines with the conserved lysine (K375) mutant were unsuccessful, presumably because its expression is incompatible with proliferation. Cell lines constructed with the Raf-ts2 conditional dominant-defective mutant displayed somewhat leaky growth phenotypes: incomplete cessation of growth at the restrictive temperature and slightly impaired growth at the permissive temperature. These phenotypes are consistent with the *in vitro* kinase activity of the ts2 mutant protein. Nevertheless, the cell lines are stable and can be propagated extensively at the permissive temperature, and they display up to 80% inhibition of exit from G₁/S at the restrictive temperature. The ts2 mutant protein accumulated in its low-mobility form on SDS-PAGE at the restrictive temperature, a condition under which MAPK pathway activity is reduced. The retarded form of Raf-1 is believed to be generated by an inhibitory phosphorylation that constitutes a feedback mechanism. At face value, the data presented here would argue against such a mechanism; however, the hyperphosphorylation of Raf-1 is poorly understood, and it will, therefore, be of interest to investigate why and how this effect occurs in the ts2 cell line.

Expression of the dominant-defective Raf-ts2 protein had profound and rapid consequences on the activity of the major known Raf target, the MAPK pathway. Up-shift to the restrictive condition at the time of serum stimulation did not abrogate the initial stimulation of the MAPK pathway, but the activity of the pathway decayed rapidly and remained at a low level. The initial stimulation of MAPK activity may reflect the need for the mutant protein to accumulate in its inactive conformation to compete effectively with the endogenous pool of wild-type c-Raf-1. Shifting cells to the restrictive temperature prior to stimulation, as well as substitution of PDGF for whole serum, did not eliminate the initial stimulation of the MAPK pathway. This may be explained by the observation that the ts2 protein is destabilized at the restrictive temperature and turns over more rapidly. The fact that the same effect was seen following PDGF stimulation suggests that the ts2 protein imposes a Raf-1 block that is intrinsically leaky.

A leaky G₁/G₂ defect could be manifested in a number of ways. First, exit could be impaired, such that cells would exit gradually over a prolonged period of time. Second, progression could be impaired, such that cells would exit synchronously but would progress toward S phase over an extended period of time. Third, the culture could be composed of two distinct populations of cells: one that was defective for exit and was permanently arrested and a second (smaller) population that was apparently normal and accomplished the G₁→S transition in synchrony with normal cells. Flow cytometric analysis of Raf-ts2 cell lines point to the last explanation: the shapes of the G₁ and S peaks were identical for mutant and control cell lines, the only difference being a reduced amplitude of the mutant peaks. The same phenom-
The expression level of the dominant-defective Raf-ts2 protein in the Rf6 cell line is apparently very close to this threshold level, such that small differences among individual cells can result in two populations of exit-deficient and exit-competent cells.

The phenotypes of both the conditionally active and the conditionally dominant-defective mutants point to a critical function for Raf-1 at very early times of exit from G2 and entry into G1. First, experiments with conditionally active mutants indicate a downstream function in the PDGF and EGF pathways, leading to the establishment of competence for cell cycle entry. Robust entry into S phase is shown to require, in addition, the progression faction IGF-1. Whether the entire competence-promoting function of the PDGF signal is mediated by Raf and the MAPK pathway remains to be investigated. Second, experiments with conditionally dominant-defective mutant indicate a defect in the early activation of the MAPK pathway. The requirement for Raf-1 activity at other points of the cell cycle is currently under investigation.

Materials and Methods

Cell Lines and Culture Conditions. The sources of the cell lines TGR-1 and NIH-3T3 have been described (59). TGR-1 is an hprt- subclone of the Raf-1 cell line (59). The ecotropic retrovirus vector packaging cell line V' (3) was obtained from Richard Mulligan. Cells were cultured in DMEM (containing glutamine, pyruvate, high glucose, and 3.7 g/liter sodium bicarbonate), supplemented with penicillin-streptomycin and 10% CS, in a 5% CO2 atmosphere at the indicated temperatures. The actual temperature of the medium inside culture vessels was monitored using a digital thermometer equipped with a probe. All drug selections were performed by feeding cells at 30% confluence immediately prior to selection. The Rf5 and Rf6 cell lines were selected with 10 μg/ml (w/v total drug) of G418 (the lot of G418 that was used contained 53% active drug). To assay anchorage-independent growth (soft agar growth assays), plates were incubated for 3 weeks (feeding was at 6-day intervals), and macroscopic colonies were counted on photographic enlargements.

Growth Rate Determination. Exponentially growing cells at 34°C were harvested with trypsin and plated into six-well microtiter plates. After 12–24 h of incubation at 34°C, the first time point was harvested to determine the initial cell number. Cell numbers were determined by trypaning individual cells and counting cells in a Coulter Counter. Three separate wells were counted for each data point. The first time point was designated t = 0 h, at which time some plates were shifted to the restrictive temperature. Cell density at the final time point did not exceed 50% confluence.

Cell Cycle Analysis. Cells from continuously growing cultures were seeded at 20–30% confluence and incubated 24–36 h until they reached 70–80% confluence. Cultures were made quiescent by reducing serum supplementation to 0.25% CS, and incubation was continued for 38–48 h. Cells expressing the wild-type EC12 protein cannot be made quiescent. To induce cell cycle entry, the medium was removed and replaced with prewarmed fresh medium containing 10% CS. In some experiments, cells were stimulated with prewarmed fresh medium containing 0.25% CS and the indicated concentrations of purified growth factors (Life Technologies, Inc.) and defined recombinant PDGF (BB homodimer), human recombinant IGF-1, and murine EGF (purified from submaxillary gland). Stimulation with prewarmed fresh medium containing 0.25% CS only did not induce entry into S phase. The temperatures at which growth of cells, serum starvation, and serum stimulation were performed were indicated above. Flow cytometry was performed as described (60). To measure [3H]thymidine incorporation, cells were grown in 24-well microtiter plates and labeled with 1.5 μCi/ml [3H]thymidine (85 Ci/mmol) for the indicated times. Labeling was quenched by the addition of ascorbic acid (50 μM). Each well was washed with three changes of ice-cold PBS (1 ml, 5 min each wash). Cells were extracted with two changes of ice-cold 5% TCA (1 ml/well, 30 min each wash). The contents of each well were dissolved in 0.5 ml of 1 N NaOH (1 h at 60°C). Samples were neutralized by addition.

---

**Fig. 10.** The effect of the dominant-defective Raf-ts2 protein on the G2–S transition. A–D, cultures were serum deprived for 48 h at the permissive temperature and stimulated with fresh medium containing 10% CS at the zero time point (t = 0 h). A and C, incubation was continued at the permissive temperature. B and D, cultures were shifted up (at t = 0 h) to the restrictive temperature. Samples were harvested at the indicated times (h) and processed for flow cytometry. Cells were stained with propidium iodide, and cell cycle analysis of the DNA histograms was performed using the mathematical model of Jett (68). A minimum of 10,000 cells were collected for each data point. Data points, percentage of total for each phase of the cycle. A and B, G2/G1 phase content. C and D, S-phase content. An individual representative experiment is shown; the results were reproducible in two independent trials. E and F, cultures were serum deprived for 48 h at the permissive temperature, stimulated with recombinant PDGF at the zero time point (t = 0 h), and shifted up to the restrictive temperature (40.5°C) at the same time. In E, cells were subjected to various concentrations of PDGF, as indicated. Titrated thymidine was added at t = 0, and labeling was continued for 24 h, at which time samples were harvested and processed for TCA-insoluble cpm. In F, cells were stimulated at t = 0 with 12.5 ng/ml PDGF, samples were labeled at the indicated times for 1 h, harvested, and processed for TCA-insoluble cpm.
of an equal volume of 1 m glacial acetic acid and counted using Ultima Gold scintillation cocktail (Packard).

Recombinant DNA Procedures. All recombinant DNA manipulations were performed by standard procedures (61). Alanine-scanning mutations were constructed directly in the EC12 expression vector (Fig. 1) as described (62) and were verified by DNA sequencing (data not shown). Electroporation conditions of TgR-1 and NIH-3T3 cell lines were as described (59). Plasmids EC12(10) and pB4X (41) were obtained from Ulf Rapp, and pMNC-Raf1 (11) was obtained from Walter Kolch.

Immunoblotting Analysis. Cells were grown at the indicated temperature to 80–90% confluence and were harvested by rapid lysis in Laemmli sample buffer (54). Laemmli sample buffer was supplemented with protease inhibitors (10 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM PMSF). Equal loading of lanes was established as described (54), except that Coomassie blue-stained gels were quantitated using the Gel Doc digital gel documentation system and Molecular Analyst software (Bio-Rad). Gels were transferred to Immobilon-P membranes (Millipore), and the immunoblots were probed using standard procedures (54, 63). Signals were visualized with the ECL system (Amersham). The monoclonal anti-c-Raf-1 antibody PBB1 (64) was provided by Ulf Rapp. The phosphspecific MAPK and MEK antibodies were purchased from New England Biolabs.

CAT Assays. NIH-3T3 cells were electrophoresed with the indicated plasmid DNAs, and equal amounts of protein were assayed for CAT activity as described (65, 66). All electroporations contained 5 μg of reporter plasmid pB4X and 50 μg of EC12, EC12-ts1, EC12-ts2, or empty vector plasmids. In each electroporation experiment, the requisite number of dishes was harvested (two per electroporated sample), and the harvested cells were pooled, centrifuged, and washed in transfection buffer. Pooled cells were divided into the requisite number of aliquots, which were electroporated in rapid succession and immediately replated in prewarmed medium. Using this procedure, electroporation efficiencies are essentially identical among different samples in a single experiment. Total protein content of the extracts was measured with the micro-bicinchoninic acid assay (Pierce), and equal amounts of protein were processed for determination of CAT activity.

Kinase Assays. Cells were grown to confluence in 10-cm dishes at the permissive temperature, rinsed with PBS, and lysed in 2 ml of ice-cold radioimmunoprecipitation assay buffer [10 mM Na2HPO4 (pH 7.0), 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 2 mM EDTA, 200 μg/ml Na3VO4, 1 mM PMSF, and 10 μg/ml aprotinin]. One ml of the extract was immunoprecipitated with 2 μl of cRaf-VI antisera (66, 67) and 20 μl of protein-A agarose beads (Boehringer Mannheim) for 2 h at 4°C. Immunoprecipitates were washed twice with radioimmunoprecipitation assay buffer, twice with PAN [10 mM Pipes (pH 7.0), 100 mM NaCl, 10 μg/ml aprotinin, and 1 mM PMSF] supplemented with 0.5% Nonidet P-40, and twice with PAN. Beads were resuspended in 10 μl of PAN, and 25 μl of kinase buffer [20 mM Pipes (pH 7.0), 10 mM MgCl2, 10 μg/ml aprotinin, and 1 mM PMSF] was added. The kinase reaction was initiated by addition of cold ATP to 2 μM, 20 μCi of γ-ATP (6000 Ci/mmol), and 70 ng of his-tagged recombinant kinase-inactive MEK (68). The total reaction volume was 40 μl. Incubation was at 23°C or 30°C for various times. Raf-EC12 (wild-type) activity was thermosensitive in vitro at temperatures above 30°C (data not shown). MEK phosphorylation activity contributed by endogenous c-Raf-1 was insignificant due to the very high levels of expression of the EC12 proteins (data not shown). Reactions were terminated by addition of 3× Laemmli SDS-PAGE sample buffer and boiling. Samples were resolved by SDS-PAGE and blotted onto Immobilon P membrane (Millipore). Radioactivity in MEK bands was quantitated by Phosphorimage analysis (Fuji). Membranes were subsequently immunoblotted with the PBB1 antibody (64) to control for the amount of Raf protein present in each reaction. ECL signals on X-ray films were quantitated using the Gel Doc system and Molecular Analyst software (Bio-Rad).

Acknowledgments

We gratefully acknowledge Walter Kolch and Harald Mischak for providing us with purified recombinant MEK protein, cRaf-VI antisera, and advice on Raf kinase assays. We thank Ulf Rapp for providing Raf monoclonal antibody PBB1 and plasmids EC12 and pB4X.

References


