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Ras-Mediated Phosphorylation of a Conserved Threonine Residue Enhances the Transactivation Activities of c-Ets1 and c-Ets2

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The Ras oncogene products regulate the expression of genes in transformed cells, and members of the Ets family of transcription factors have been implicated in this process. To determine which Ets factors are the targets of Ras signaling pathways, the abilities of several Ets factors to activate Ras-responsive enhancer (RRE) reporters in the presence of oncogenic Ras were examined. In transient transfection assays, reporters containing RREs composed of Ets–AP-1 binding sites could be activated 30-fold in NIH 3T3 fibroblasts and 80-fold in the macrophage-like line RAW264 by the combination of Ets1 or Ets2 and Ras but not by several other Ets factors that were tested in the assay. Ets2 and Ras also superactivated an RRE composed of Ets-Ets binding sites, but the Ets-responsive promoter of the c-*fms* gene was not superactivated. Mutation of a threonine residue to alanine in the conserved amino-terminal regions of Ets1 and Ets2 (threonine 38 and threonine 72, respectively) abrogated the ability of each of these proteins to superactivate reporter gene expression. Phosphoamino acid analysis of radiolabeled Ets2 revealed that Ras induced normally absent threonine-specific phosphorylation of the protein. The Ras-dependent increase in threonine phosphorylation was not observed in Ets2 proteins that had the conserved threonine 72 residue mutated to alanine or serine. These data indicate that Ets1 and Ets2 are specific nuclear targets of Ras signaling events and that phosphorylation of a conserved threonine residue is a necessary molecular component of Ras-mediated activation of these transcription factors.

The Ras gene products are GTP-dependent molecular switches essential for growth of cultured cells (3, 45). The Raf proto-oncogene product directly interacts with Ras in a GTP-dependent manner (47, 48, 56), identifying a long-sought effector of Ras action. The Raf kinase can activate MEK kinases, which in turn activate the mitogen-activated kinase (MAP) kinase family (13, 14, 24). Activation of this protein kinase cascade is critical for Ras to exert effects on differentiation and cell growth (10, 36). Among the targets of Ras-triggered protein kinase cascades in cells are nuclear transcription factors which regulate genes that ultimately confer specific cellular phenotypes (reviewed in references 5, 20, and 27). Two families in particular, the Ets and AP-1/ATF families of transcription factors, have been identified as key nuclear mediators of Ras action.

The Ets family of transcription factors are related to the oncogene first identified in the chicken E26 retrovirus and share a highly conserved DNA-binding motif termed the Ets domain (26). The Ets factor Elk-1/SAP-1 regulates the c-*fos* promoter through formation of a ternary complex with the serum response factor (11). Elk-1 is a target of MAP kinases p42 and p44, and phosphorylation of the C-terminal portion of the protein leads to a stimulation of transactivation by the Elk-1–serum response factor complex (23, 35). Likewise, the

Drosophila Ets factor Pointed has recently been shown by genetic criteria to be a direct target for Ras-MAP kinase signaling during eye development in the fly, although actual gene targets of this factor have not been identified (8, 28, 38). In addition to the Ets domain, Pointed contains a region of approximately 100 amino acids, termed the Pointed domain, that is highly conserved within a subgroup of Ets factors, in particular with Ets1 and Ets2 (29, 52). This Pointed domain region contains a threonine residue at position 151 that is phosphorylated in vitro by *Drosophila* MAP kinase (8), and a Pointed protein engineered to contain an alanine residue at threonine 151 could not transactivate a reporter gene in a Ras-dependent fashion (8, 38).

Both of the AP-1 family members c-*fos* and c-*jun* encode products whose transactivation potential can be enhanced by Ras via a molecular mechanism that depends on phosphorylation of specific residues in these transcription factors (4, 12, 44). Kinases that mediate the phosphorylation of c-Jun (JNK kinases) have been identified and molecularly cloned. These are members of the MAP kinase superfamily but distinct from the best-characterized members of the MAP kinase family, pp42 and pp44 (15, 22), and apparently regulated by a distinct effector pathway as well (37). The Ras-activated kinase that stimulates the activity of the transcription factor c-Fos appears to be distinct from JNK or MAP kinases p42 and p44 (12). In addition, the CREB/ATF factor has been demonstrated to be a target of Ras signaling in PC12 cells via an apparently unique kinase (19). Thus, Ras activation of Elk-1 and AP-1/ATF transcription factors depends on different effector pathways con-

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sisting of related but distinct protein kinases. The bifurcation of Ras effectors and signaling is likely to at least partially account for the disparate effects attributed to Ras both during cell transformation and differentiation (36).

In addition to stimulating immediate-early gene expression, Ras signaling can potentiate the stable expression of genes in transformed cells or when activated by growth factor receptors such as c-Fms. One class of enhancer element that is a target for this type of prolonged Ras action is composed of binding sites for Ets family members and AP-1/ATF family members (5, 20). Dominant negative versions of c-Jun or c-Ets2 block Ras mitogenic signaling (32, 34, 51), demonstrating that these families of factors are necessary for Ras transformation. In the case of the Ets2 dominant negative gene, signaling emanating either from an activated *ras* oncogene or from c-*fms* activation of endogenous Ras is affected (34). Many cellular genes have now been demonstrated to contain a Ras-responsive enhancer (RRE) composed of binding sites for both classes of these nuclear factors (5, 20), and activation of such genes likely plays a role in a diverse array of cellular processes (5).

As discussed above, AP-1/ATF family members are targets for Ras signaling pathways and are likely to be involved in regulating genes containing the composite Ets–AP-1 motif. In transient assays, the Ets factors Ets1 and Ets2 have been shown to collaborate with AP-1 factors in the activation of a reporter that contained multiple copies of the polyomavirus (Py) oncogene-responsive enhancer (49). Additionally, in the proper promoter context, Ets2 activation is enhanced by coexpression of activated Ras (18, 53). Recently, it has been demonstrated that the p68 form of Ets1 interacts with the POU homeodomain transcription factor PIT-1 to stimulate Ras-enhanced activity of the prolactin promoter (7). However, there have been no published reports concerning the mechanism of Ets activation in mammalian cells. In the present report, we show that Ets1 or Ets2 selectively collaborated with Ras in the activation of Ets–AP-1 composite responsive elements, and Ets2 also activated an element composed of a palindromic Ets-Ets composite site. Mutation of a critical threonine residue within the amino-terminal domain conserved in Ets1 and Ets2 results in abrogation of Ras and Ets synergism. Finally, we provide biochemical evidence indicating this threonine residue is phosphorylated in response to Ras signaling. These results define a molecular determinant in the Ets1 and Ets2 proteins necessary for Ras action in establishing altered patterns of gene expression.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis. The reporter plasmids for NVL3 (39), urokinase-type plasminogen activator (uPA) (46), Py enhancer, and E.18 (18) have been previously described. The expression vectors for activated *ras* were also previously described (18, 39). The human c-*fms* promoter used contains 433 bp of sequence adjacent to the first ATG residue in exon 2 of the gene and was constructed as described previously (40).

The human Ets2 influenza virus hemagglutinin-tagged vector and the vector encoding the p54 form of chicken Ets1 have been previously described (40). The FNpcDNA3 expression vector was constructed by using the cytomegalovirus promoter-driven expression vector pcDNA3 (Invitrogen, San Diego, Calif.). Sequences that encoded the FLAG epitope tag, recognized by the FLAG M2 monoclonal antibody (Kodak/IBI, New Haven, Conn.), and the simian virus 40 T-antigen nuclear localization signal were inserted between the *Hin*dIII and *Bam*HI sites. The Ets2 coding sequence was directionally subcloned into this vector as a *Bam*HI-*Sal*I fragment following addition of an in-frame *Bam*HI site at the 5′ end and a *Sal*I site just 3′ of the termination codon. In all of the transactivation assays, unmodified Ets2 expressed from the parent vectors behaved in the same manner as the epitope-tagged versions of Ets2 (data not shown).

The mutations in p54 Ets1 and the hemagglutinin-tagged Ets2 expression vectors were introduced by a PCR strategy that depended on the unique *Hpa*I site which included the codon for threonine 38 or threonine 72. One PCR primer containing this *Hpa*I site with the appropriate base change to alter the coding

sequence from threonine to different amino acids was used in combination with a primer located 3′ that encompassed either the *Eco*RV site or *Xmn*I site uniquely located in the *ets1* or *ets2* cDNA, respectively. Introduction of the mutations resulted in the loss of the *Hpa*I site. Mutations of FN-Ets2 constructs that alter residue 72 were constructed by using a Chameleon site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) as recommended by the manufacturer.

The expression vectors for human Fli-1 (30) and mouse Elf-1 (33) were previously described. The cDNA for murine PEA3 (54) was subcloned into the hemagglutinin-tagged vector used for human Ets2 expression (see above).

Transfection assays. NIH 3T3 cells were grown in culture and transfected by the calcium phosphate method exactly as described previously (18, 39). Growth and electroporation of RAW264 cells were accomplished exactly as described previously (46). For these experiments, expression vectors for Ets factors and Ras were titrated to determine the level of plasmid that saturated reporter activity. The amounts of plasmids used for the assays presented here including the superactivation assays, were set at the saturating level. In general, these amounts were 0.1 μg for Ets2 and other factors expressed using cytomegalovirus-based vectors and 1 μg for Ets1 or Ras.

Immunoprecipitation and phosphoamino acid analysis. NIH 3T3 cells were cotransfected with 5 μg of plasmid expressing wild-type or mutant FLAG epitope-tagged Ets2 protein and either 2 μg of oncogenic Ras expression vector or 2 μg of empty expression vector. At 20 h after addition of the calcium phosphate precipitate, the medium (Dulbecco modified Eagle medium containing 10% calf serum) was removed and replaced with Dulbecco modified Eagle medium containing 0.5% calf serum. The cells were then incubated for another 20 h. This treatment following transfection was the same as that used for transactivation analysis with the E.18 reporter construct. The serum-starved cells were then washed and refed with phosphate-free medium containing 2 to 3 mCi of $^{32}P_i$ per ml. In the parallel experiments to analyze protein expression levels, the cells were refed with methionine-free medium containing 200 μCi of [^{35}S]methionine per ml. After 4 h of incubation, the cells were washed and lysed with radioimmunoprecipitation assay buffer for 20 min at 4°C. The extract was collected and precleared with rabbit preimmune serum for 1 h. The precleared extract was then incubated with 8 μg of anti-FLAG M2 monoclonal antibody (IBI/Kodak) for 2 h, and the antibody-antigen complex was precipitated with protein G-coated Sepharose beads (GammaBind Plus; Pharmacia). The samples were separated on sodium dodecyl sulfate 9% (SDS)–polyacrylamide gels, and the gel with the ^{35}S-labeled samples was dried and analyzed by with a phosphoimager (Bio-Rad, Richmond, Calif.).

The gel containing the ^{32}P-labeled samples was electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.), and the Ets2 band was cut out and incubated in 50 μl of 6 N HCl for 80 min at 100 to 110°C. The samples were diluted in deionized water and dried under vacuum. Phosphoamino acid analysis was performed as described previously (6). Briefly, the samples were resuspended in formic acid-acetic acid buffer (pH 1.9). They were then spotted onto cellulose plates, along with 1.0 μg each of phosphothreonine, phosphoserine, and phosphotyrosine standards, and run on a Hunter thin-layer electrophoresis unit (HTLE 7000; CBS Scientific, Inc., Del Mar, Calif.). The samples were separated in the first dimension at pH 1.9 for 20 min at 1.5 kV and then in the second dimension at pH 3.5 for 16 min at 1.3 kV. The standards were visualized by spraying with 0.25% ninhydrin in acetone. The ^{32}P-labeled amino acids were visualized and quantitated with a phosphoimager. The data were quantitated and displayed with Molecular Analyst software obtained from Bio-Rad.

RESULTS

Superactivation of Ets–AP-1 RRE reporters by the combination of Ras and Ets1 or Ets2. The isolated DNA binding domain of human c-Ets2 abolishes mitogenic signaling by Ras or c-Fms and also blocks activation of downstream genes that contain RREs composed of binding sites for Ets factors and AP-1 (32, 51). While these data demonstrate the importance of Ets family members in Ras signaling events crucial for cell transformation, they do not identify which Ets family members are involved because of the similarity in DNA binding sites for Ets family proteins. To address this issue, we decided to identify which Ets family members could be activated by Ras. For these experiments, we used five different reporter constructs in transient transfection assays in NIH 3T3 fibroblasts (Fig. 1). These reporters were transfected along with an expression vector for Ras, Ets1, or Ets2 or the combination of Ras with either Ets1 or Ets2.

The NVL3 RRE is a prototype viral RRE that contains an Ets–AP-1 composite site (39). This enhancer (Fig. 1A) was weakly activated by human Ets2 (two- to threefold induction) or the p54 form of chicken Ets1 (four- to fivefold induction).
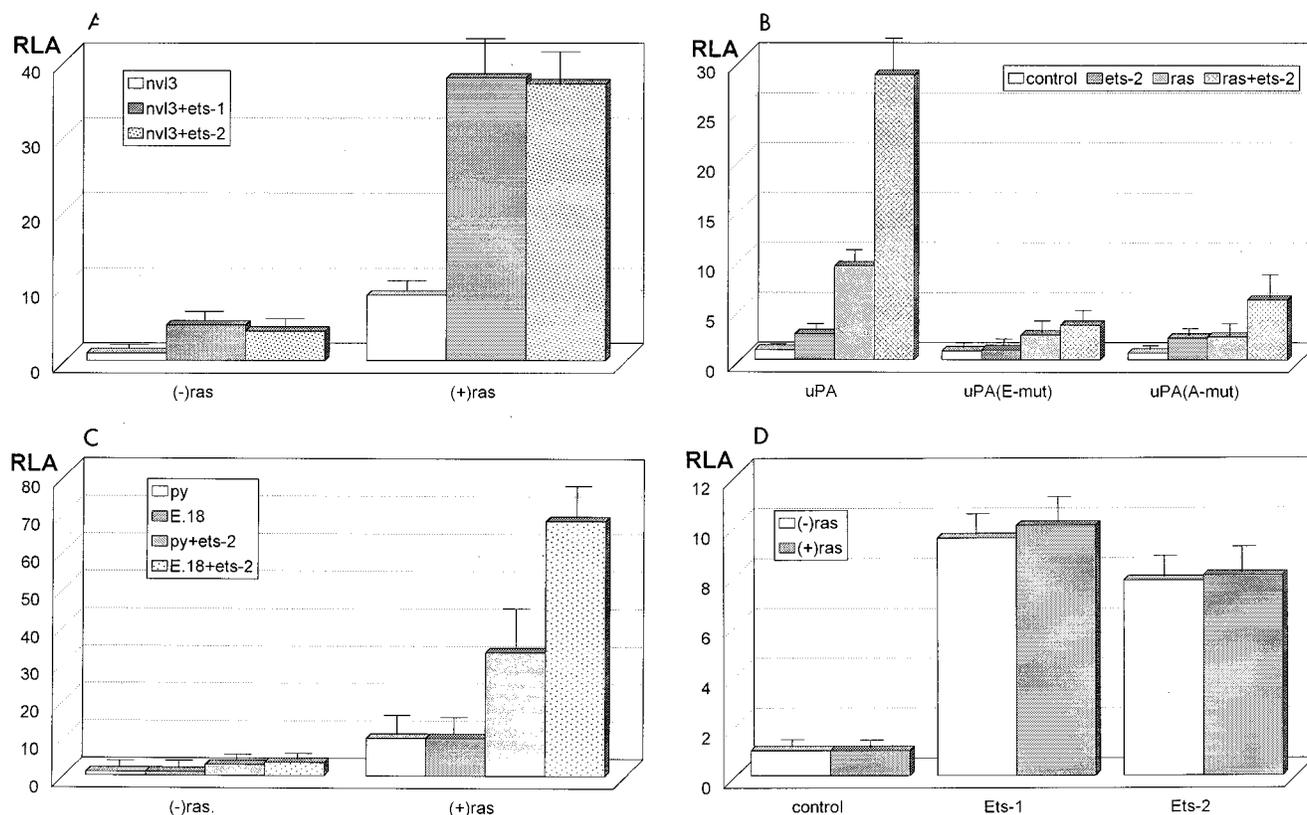
FIG. 1. Superactivation of RRE reporters by the combination of Ras with c-Ets1 or c-Ets2. (A) Activation of the NVL3 RRE luciferase reporter by c-Ets1 and c-Ets2 (pGCN-E2) in NIH 3T3 cells in either the absence [(−)ras] or presence [(+)ras] of activated human Ha-c-Ras (1 μg). (B) Activation of uPA luciferase reporters in NIH 3T3 cells by c-Ets2 (pGCN-Ets2), Ras, or the combination of the two, as indicated. The reporters used contained wild-type Ets–AP-1/ATF elements (uPA, AGGAAATGAGGTCA), Ets site mutated [uPA(E-mut), AccAAATGAGGTCA], and AP-1/ATF site mutated [uPA(A-mut), AGGAAATGAGGagA]. (C) Activation of the Py and E.18 CAT reporters by c-Ets2 (FN-E2) in the absence [(−)ras] or presence [(+)ras] of Ras. The E.18 site consists of an inverted repeat of an Ets motif (GACCGGAACTACTTCCGGTC). (D) Activation of the c-fms proximal promoter in NIH 3T3 cells by Ets1 and Ets2 in the absence or presence of Ras, as indicated. The −430 human c-fms promoter (40) was used for these experiments. For all experiments, the averages of four separate experiments performed in duplicate are presented, and error bars indicate standard deviations. For these experiments, the activity of the reporter in the presence of empty expression vectors (control lane) was set to 1. Activity is expressed as relative luciferase activity (RLA).

As expected, the NVL3 RRE was strongly responsive to activated Ras (10-fold induction). However, the combination of Ets1 or Ets2 and Ras stimulated NVL3 RRE activity 35- to 40-fold. (Fig. 1A). This effect, which we term superactivation, was clearly more than the result of a simple additive effect of Ets1 or Ets2 and Ras. When a farnesylation-negative Ras mutation was used, no collaboration with Ets factors was observed (data not shown). For the experiments using Ets2, a form of the protein that was epitope tagged at the amino terminus with an influenza virus-derived peptide was utilized (PGCN-E2; see Materials and Methods). Identical results were obtained with the human Ets2 cDNA without the epitope tag (data not shown).

The cis sequence requirement for Ras-Ets superactivation in a cellular gene was determined by using the uPA promoter (Fig. 1B). This promoter contains an Ets–AP-1/ATF motif that is located approximately 2,300 bp upstream of the transcription initiation site of the promoter and has been previously identified as an element that responds to phorbol esters or other growth factors such as epidermal growth factor (41) and colony-stimulating factor 1 (46) and can also be transactivated by coexpression of Ets2 (46). For the experiments presented in Fig. 1B, a short DNA fragment containing the distal Ets–AP-1/ATF element was placed immediately upstream of the proximal uPA promoter at position −110 (41), and this combina-

tion was linked to a firefly luciferase reporter gene. Enhancer elements that contained point mutations in either the Ets-related or AP-1-related sites (see Materials and Methods) were also tested for Ras and Ets responsiveness. The data indicated that the uPA distal element behaved as an RRE in these assays and that the activity of this RRE depended on the integrity of both Ets and AP-1/ATF sites (Fig. 1B). Additionally, the native uPA RRE was superactivated nearly 30-fold by the combination of Ras and Ets2. When Ets1 was substituted for Ets2, a similar result was obtained (see below). The superactivation by Ras and Ets2 depended on intact Ets and AP-1/ATF binding sites, because the reporters containing mutations in either of these sites were no longer superactivated. Similar results were obtained with a reporter construct that contained 4 kb of the proximal uPA promoter with the RRE in its endogenous position at −2300 (data not shown).

The stromelysin promoter contains an RRE composed of two inverted Ets sites (50), and an artificial construct based on this type of element, termed E.18, is a robust RRE reporter (18). The superactivation of this element was determined in parallel with the Ets–AP-1 Py prototype oncogene-responsive enhancer (49). One copy of either of these elements was placed in the identical reporter context (18). For these experiments, a FLAG epitope-tagged form of Ets2 (FN-E2; see Materials and Methods) was employed, but identical results were obtained
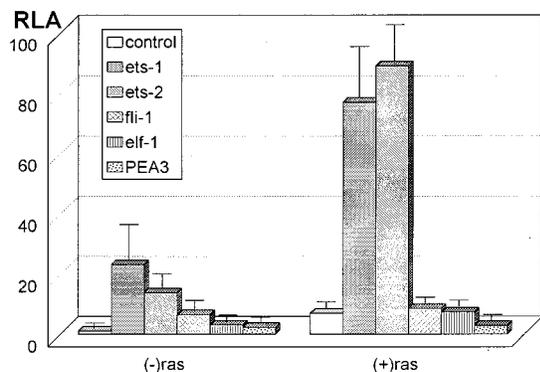
FIG. 2. Superactivation of RRE activity by Ras and Ets factors is selective. The activity of the uPA luciferase reporter was measured in RAW264 cells in the presence of Ets1, Ets2 (PGCN-E2), Fli-1, Elf-1, or PEA3, as indicated, in the absence [(−)ras] or presence [(+)ras] of Ras. The averages of four experiments performed in duplicate are presented, and the error bars indicate standard deviations. The activity of the reporter in the presence of empty expression vectors (control lane) was set to 1. RLA, relative luciferase activity.
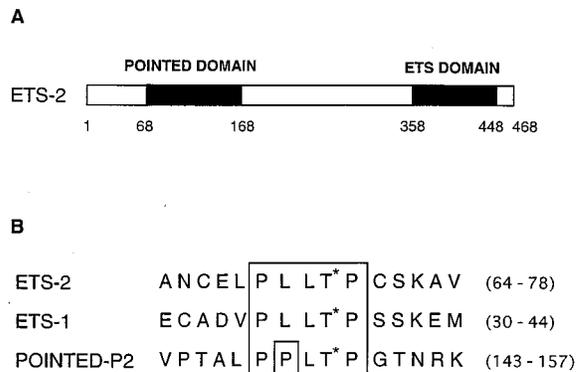

FIG. 3. An N-terminal domain conserved in c-Ets1 and c-Ets2. (A) Illustration of the Ets2 protein, with the amino-terminal conserved Pointed domain as well as the carboxy-terminal Ets domain highlighted. (B) Comparison of the sequences of Ets2, Ets1, and Pointed surrounding the putative kinase substrate region. The conserved threonine residue, a potential MAP kinase site, is indicated by ∗.

when an untagged Ets2 expression construct was used (data not shown). The results obtained with the Py-chloramphenicol acetyltransferase (CAT) reporter indicated that activation by Ets2 or Ras alone and superactivation by the combination of the two genes was virtually identical to what was seen with the other two Ets–AP-1 reporters (3-, 10-, and 30-fold activation, respectively; Fig. 1C). Ets1 did not efficiently activate the E.18 reporter either alone or in combination with Ras (data not shown). However, the E.18 reporter was activated by Ets2 or Ras to roughly the same extent but was superactivated by the combination over 60-fold (Fig. 1C). Thus, either an Ets–AP-1 or Ets-Ets RRE element could be superactivated by Ras and Ets-factors.

The murine and human c-*fms* promoters are transactivated by Ets1 and Ets2, and an Ets2-responsive binding site in the human promoter has been identified (40, 42). For these experiments, we cotransfected the human c-*fms* macrophage-specific promoter located in the first intron of the gene along with Ets and Ras expression vectors (Fig. 1D). This promoter contained 430 bp of information located adjacent to the first coding intron of the gene including the site identified as an Ets binding site (40). As described previously, this promoter responded to cotransfection of an Ets1 or Ets2 expression plasmid with an 8- to 10-fold increase in reporter gene expression in NIH 3T3 fibroblasts. Cotransfection of activated Ras had no effect on either basal or Ets-stimulated activity (Fig. 1D). The same results were obtained when the macrophage cell line RAW264 was used (data not shown). The data demonstrate that the c-*fms* Ets-responsive element is not an RRE.

Electrophoretic mobility shift assays (EMSA) using recombinant Ets2 protein and the binding sites from the uPA or E.18 reporter have demonstrated that Ets2 can directly interact with these RREs (46). Likewise, recombinant Ets2 can bind to the NVL3 RRE in vitro (data not shown). The EMSA results are consistent with a direct in vivo interaction between Ets1 or Ets2 and the various RREs used in the experiments described above.

Selective superactivation of Ets1 and Ets2 by Ras. To determine whether the effect of Ras and Ets1 or Ets2 was selective or a general property of the overexpression of Ets factors, the abilities of other Ets family members, including human Fli-1, mouse Elf-1, and mouse PEA3, to superactivate RRE reporters were tested (Fig. 2). The experiments reported here

were conducted in the murine macrophage cell line RAW264. These cells have 5- to 10-fold lower c-Ets2 mRNA than NIH 3T3 cells (data not shown). Consistent with the lower levels of endogenous Ets2 mRNA, both Ets1 and Ets2 strongly activated the uPA reporter (20- and 13-fold activation, respectively). Strikingly, the combination of Ets1 and Ets2 with Ras activated this reporter 80- to 100-fold. In contrast, Fli-1 activated the reporter sixfold, while Elf-1 or PEA3 activated the uPA reporter only two- to threefold, and none of these factors displayed superactivation in combination with Ras; in fact, PEA3 partially blocked Ras activation of the uPA reporter. Results similar to those represented in Fig. 2 were obtained for NIH 3T3 cells when either the uPA or NVL3 reporter was used (data not shown). It is unlikely that the lack of Ras superactivation of PEA3 or Elf-1 was due to their inability to bind RREs, because when these proteins were expressed in COS cells, EMSAs with RRE probes revealed that new specific complexes were formed (data not shown). Analysis of steady-state expression of the epitope-tagged version of the PEA3 and Ets2 proteins used in these experiments (see Materials and Methods) indicated that the two proteins were expressed at the same level, indicating that for PEA3, lower levels of protein expression are not the reason for the absence of Ras responsiveness. Together, the results indicated that the superactivation of reporters by Ets1 or Ets2 and Ras is highly selective.

In addition to the Ets domain, which is 95% conserved between Ets1 and Ets2, these two proteins also share a region of homology of over 100 amino acids present in their amino-terminal regions (52) (Fig. 3A). This region of homology is also shared with the *Drosophila* Ets family member Pointed and has been termed the Pointed domain (29). Within the first 30 amino acids of this region, a potential kinase phosphorylation site, PLLTP, was found in both Ets1 and Ets2 (Fig. 3B). A similar sequence (PPLTP) is found at the same position of the *Drosophila pointed* P2 product and has been shown to be functionally important (8, 38). These sites are related to the published consensus site for MAP kinases p42 and p44 (1). These observations indicate that the amino-terminal sequence PLLTP may be critical for common behavior of Ets1 and Ets2 in the transient Ras superactivation assays (see above).

Mutation of the potential phosphorylation site threonine 38 in Ets1 or threonine 72 in Ets2 abrogates Ras superactivation. To test the importance of the potential MAP kinase phosphorylation site in Ets1 and Ets2 in the Ras superactivation of RRE

reporters, the threonine residue within this site was mutated to an alanine residue by PCR-based site-directed mutagenesis (see Materials and Methods). This threonine residue occurs at position 38 in Ets1 (p54 [52]) and at position 72 in Ets2. The expression vectors encoding these mutations were cotransfected along with the NVL3 reporter and Ras into NIH 3T3 cells (Fig. 4A and B, respectively). In each case, the single point mutations only slightly reduced the ability of Ets1 and Ets2 proteins to transactivate the NVL3 RRE. In addition, the alanine-mutated versions of Ets1 and Ets2 were able to activate the c-*fms* promoter as well as the wild-type versions of the proteins (data not shown). Conversely, the ability to collaborate with activated Ras was completely ablated. From 30- to 40-fold superactivation with wild-type Ets1 or Ets2, activation fell to the same level observed with Ras alone when the alanine 38 (Fig. 4A) or alanine 72 (Fig. 4B) mutation was tested. By analogy to other transcription factor targets of the MAP kinase superfamily, we predicted that if the Ets2 threonine 72 was substituted with a serine residue, this site of Ets2 would retain its ability to be phosphorylated. The Ets2 serine 72 protein was fully competent in superactivation of the reporter gene, and in fact, the serine 72 version was slightly more active than the wild-type Ets2 protein (44-fold superactivation, compared with 35-fold for the wild type). We also tested whether substitution of Ets2 threonine 72 with an acidic residue (aspartic acid) could partially mimic the phosphorylated threonine and lead to a higher basal level of reporter gene activation. The Ets2 aspartic acid 72 mutation created a protein with slightly higher basal-level activity (fivefold activation, compared with threefold for the wild-type Ets2 protein) but one that could not participate in superactivation (average of 11-fold activation, compared with 10-fold for Ras alone). Similar results were obtained when the uPA promoter was assayed with the Ets1 and Ets2 mutations in either NIH 3T3 or RAW264 cells (data not shown).

Superactivation of Ets-Ets RRE reporters by the combination of Ras and Ets2. To determine whether Ets2 is an independent target for Ras-mediated superactivation in the absence of an adjacent AP-1 binding site, the E.18 reporter was also used to test the Ets2 mutations in NIH 3T3 cells. The RRE in E.18 consists of two inverted Ets2 consensus binding sites and no AP-1 binding site (18). As with the NLV3 reporter, conversion of the Ets2 threonine 72 to alanine only slightly reduced responsiveness to Ets2 alone but resulted in complete loss of the previously high superactivation of the E.18 reporter mediated by Ras and Ets2 in combination (Fig. 4C). An Ets2 serine 72 factor was effective in the E.18 superactivation assay, while a glutamic acid 72 acidic residue conversion resulted in a slight increase in basal-level activation but loss of superactivation potential. The Py reporter, containing the overlapping Ets and AP-1 sites in the same context as the E.18 RRE, behaved in a similar manner with these Ets2 mutations when tested in NIH 3T3 cells (data not shown).

The absence of a significant effect of these mutations on the basal-level activity of Ets1 and Ets2 assayed on the RRE reporters as well as the c-*fms* promoter implied that the mutated proteins are expressed and folded properly. However, alterations in the level of expression, protein localization, or DNA binding of alanine-substituted Ets1 and Ets2 could still account for these results. Thus, experiments were performed to assess possible changes in these properties of the Ets2 alanine 72-mutated protein. Following transient transfection of expression constructs for either wild-type or alanine 72 Ets2 into NIH 3T3 cells, cellular proteins were metabolically labeled with [^{35}S]methionine and Ets2 expression was determined by immunoprecipitation. The epitope tag on the expressed Ets2 al-
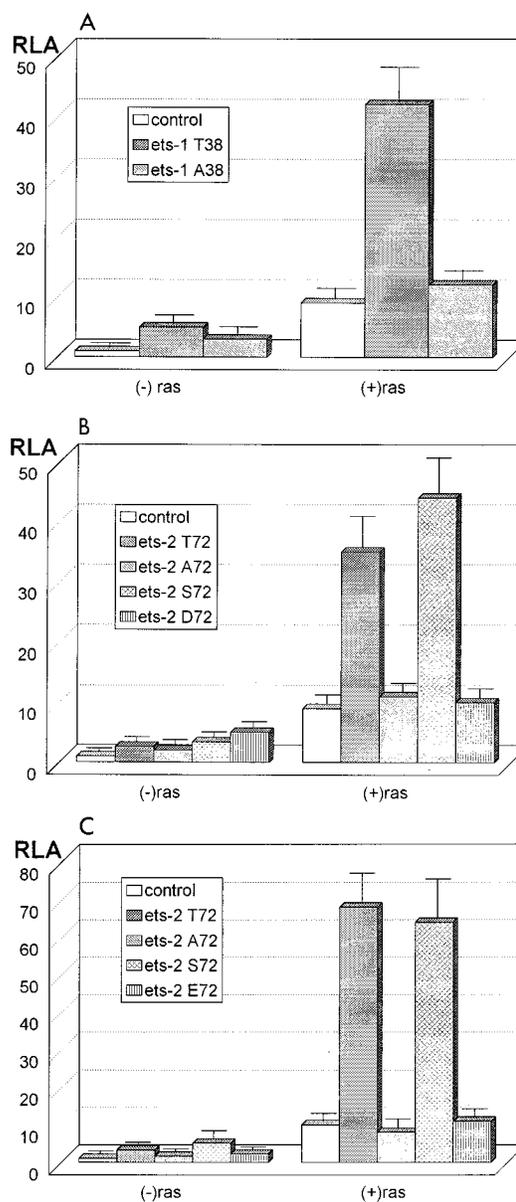

FIG. 4. Mutation of threonine 38 in Ets1 or threonine 72 in Ets2 abrogates Ras superactivation activity. (A) Activity of the NVL3 luciferase reporter in NIH 3T3 cells in either the absence [(−)ras] or presence [(+)ras] of activated Ha-c-Ras. An expression vector for wild-type threonine 38 Ets1 or mutated alanine 38 Ets1 was included as indicated. (B) Activity of the NVL3 luciferase reporter in NIH 3T3 cells in either the absence [(−)ras] or presence [(+)ras] of activated Ha-c-Ras. An expression vector for wild-type threonine 72 Ets2 (PGCN-E2) or mutated Ets2 form alanine 72, serine 72, or aspartic acid 72 was included as indicated. (C) Activity of the E.18 CAT reporter in NIH 3T3 cells in either the absence [(−)ras] or presence [(+)ras] of activated Ras. An expression vector for wild-type threonine 72 Ets2 (FN-E2) or mutated Ets2 form alanine 72, serine 72, or glutamic acid 72 was included as indicated. For all experiments, the averages of four separate experiments performed in duplicate are presented, and error bars indicate standard deviations. For these experiments, the activity of the reporter in the presence of empty expression vectors (control lane) was set to 1. Activity is expressed as relative luciferase activity (RLA).

lowed the transfected protein to be distinguished from the endogenous protein. The conditions for these experiments were the same as in the transient transfections assays (see above) and the ^{32}P labeling experiments (see below). As demonstrated in Fig. 5, activated Ras increased the expression of
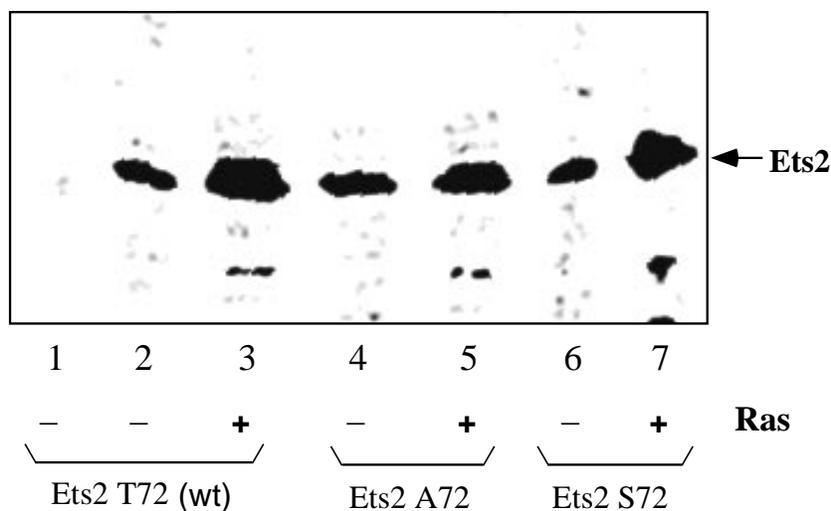
FIG. 5. Mutation of threonine 72 of c-Ets2 to alanine 72 does not grossly affect protein folding or steady-state concentrations. Comparison of Ets2 threonine 72, alanine 72, and serine 72 expressed following transient transfection of NIH 3T3 cells. Cells were transiently transfected with the indicated FN-Ets2 expression vector and, where indicated by +, an expression construct for oncogenic Ras. At 40 h after transfection, the cells were metabolically labeled with [^{35}S]methionine for 4 h, and then the exogenous Ets2 was immunoprecipitated with either nonimmune serum (lane 1) or the anti-FLAG monoclonal antibody directed against the epitope tag of Ets2 (lanes 2 to 7). The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized with a phosphoimager, and data were quantitated and displayed with Molecular Analyst software obtained from Bio-Rad. wt, wild type.

proteins from the FN-Ets2 vectors approximately threefold, but equal amounts of wild-type and alanine 72 Ets2 proteins were present in the cells, indicating that protein folding and the steady-state protein concentration were not greatly affected by mutation of residue 72. In addition, because the Ets2 alanine 72 protein did not participate in superactivation, simply increasing the amount of Ets2 protein cannot account for Ras superactivation. This interpretation is reinforced by experiments in which Ets2 protein levels were increased in the absence of Ras by simply increasing the amount Ets2 expression plasmid in the transient transfection assay. Increasing Ets2 levels three to fivefold in this way (as determined by Western blotting) did not result in superactivation of the E.18 reporter (data not shown). Immunohistochemical analysis following transient expression demonstrated that both wild-type and alanine 72 Ets2 proteins were located in the nucleus, indicating that this mutation did not affect cellular localization (data not shown). Finally, EMSA with wild-type and alanine 72 proteins expressed in COS cells or by coupled in vitro transcription-translation demonstrated that the two proteins bind the RREs with approximately equal affinities (data not shown).

Ras-dependent phosphorylation of threonine 72 in the Ets2 transcription factor. The functional data presented above demonstrated that the threonine residues present at position 72 in Ets2 and position 38 in Ets1 were critical for Ets-Ras superactivation of RRE reporters and implicated the threonine 72 and threonine 38 residues of these Ets factors as targets of Ras-dependent phosphorylation. To examine more directly the Ras-dependent phosphorylation of threonine 72 in Ets2, transfection experiments with epitope-tagged Ets2 (FN-E2) in either the presence or absence of expression of activated *ras* oncogene were performed. Transfected cells were labeled in parallel with [^{35}S]methionine or ^{32}P$_i$, and the exogenous Ets2 was immunoprecipitated (see Materials and Methods). Antibodies that recognize the FLAG epitope of the expressed Ets2 protein were used for immunoprecipitation of the Ets2 protein from the ^{32}P-labeled cell extract. The immunoprecipitate was run on an SDS-polyacrylamide gel and transferred to a polyvinylidene membrane, and the Ets2 band was identified

and eluted. Subsequently, equivalent amounts of ^{32}P-labeled Ets2 protein were subjected to phosphoamino acid analysis, and the results were analyzed on a phosphoimager (see Materials and Methods). The results of a typical experiment are presented in Fig. 6. These data demonstrated that Ets2 was extensively phosphorylated on serine residues, and comparison of Ets2 protein levels and ^{32}P incorporation (Fig. 6A and B) indicated that Ras coexpression produced no large change in total Ets2 serine phosphorylation. In contrast, phosphothreonine was not detected in Ets2 protein isolated from cells that lacked Ras expression (Fig. 6A), but threonine phosphorylation was induced by Ras coexpression (Fig. 6B). When the alanine 72 version of Ets2 was substituted for the wild-type protein in this assay, abundant serine phosphorylation was again detected independent of Ras expression. However, Ras-dependent threonine phosphorylation was no longer detected with the alanine 72 mutation (Fig. 6C and D). Further, when the serine 72 mutation, which can participate in superactivation, was examined in this assay, phosphothreonine was again not detected (Fig. 6E and F). For the serine 72 mutation, no change in Ras-dependent serine phosphorylation was detected above the high background of serine phosphorylation already present. This analysis was performed multiple times with identical results. Taken together, these data indicate that threonine 72 is the only site of Ras-dependent threonine phosphorylation present in the Ets2 protein and, consistent with the functional results presented in Fig. 4, that phosphorylation of threonine 72 is necessary for Ras activation.

DISCUSSION

The integration of signal transduction pathways and transcriptional components in the regulation of immediate-early genes has been delineated in some detail by recent work in a number of systems (23). Studies on the c-*fos* promoter provide one example in which the Ets factor Elk-1/SAP-1 has been demonstrated to be a direct target of Ras/MAP kinase p42/p44 signaling that is necessary for the transient, immediate-early transcription of c-*fos* (23). A less defined problem is how sig-
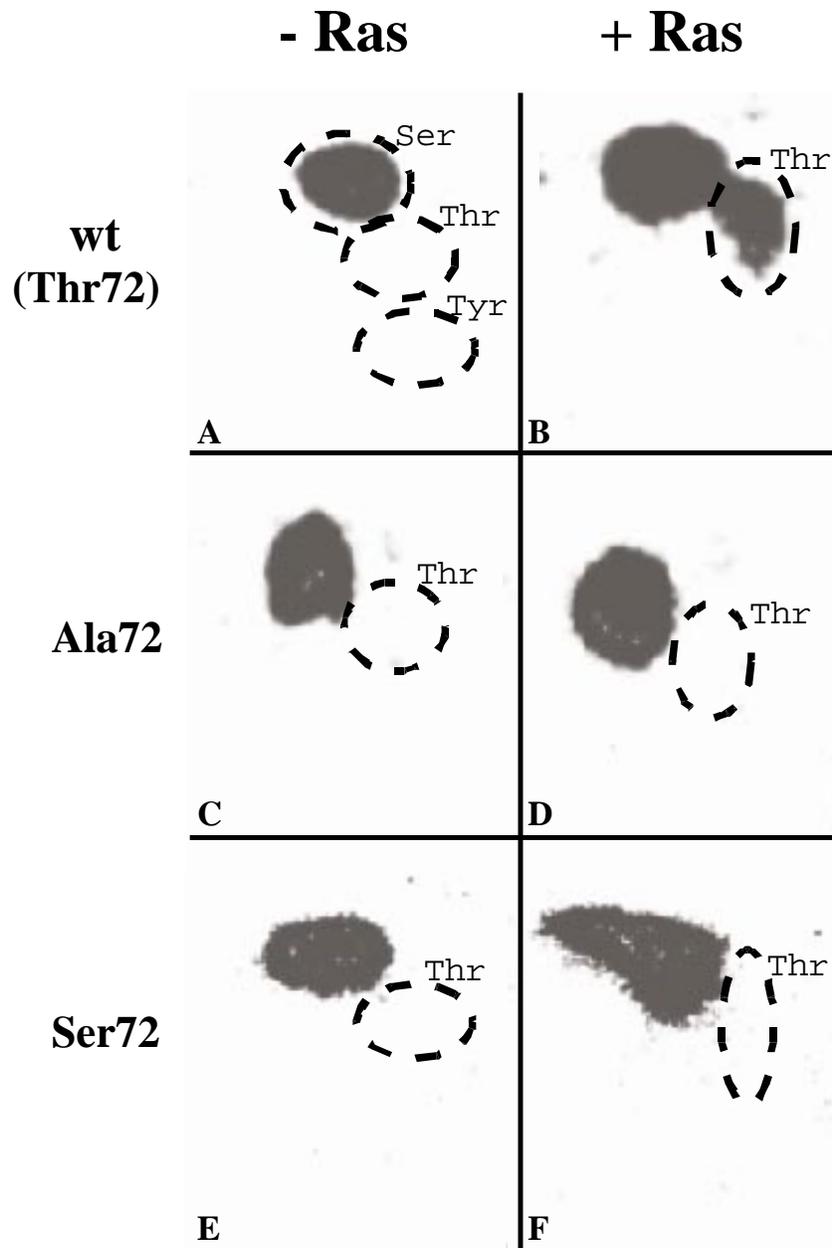
FIG. 6. Phosphorylation of threonine 72 in c-Ets2 in response to Ras signaling. An FN-E2 expression vector encoding the threonine 72 (A and B), alanine 72 (C and D), or serine 72 (E and F) version of Ets2 was cotransfected into NIH 3T3 cells with an expression construct for oncogenic Ras (+Ras) or with an empty Ras expression vector (−Ras). After 40 h, the transfected cells were metabolically labeled with $^{32}P_i$ for 4 h, and the exogenous Ets2 was immunoprecipitated with an antibody against the FLAG epitope tag. Phosphoamino acid analysis was performed as described in Materials and Methods. The dotted circles indicate in panel A the positions, following chromatography and ninhydrin detection, of the phosphoserine, phosphothreonine, and phosphotyrosine standards and in panels B to F the positions of the phosphothreonine standard. The data were quantitated and displayed with Molecular Analyst software obtained from Bio-Rad.

naling pathways lead to the stable, persistent expression of genes. The regulation of such genes is crucial to the final phenotype of cells both during terminal differentiation and during neoplastic progression. Studies involving the transcriptional enhancer elements present in genes that are regulated in response to growth factor and Ras signaling have linked Ets factors with the persistent regulation of genes in transformed cells (5, 18, 32) and during macrophage differentiation (25). In the present report, Ets1 and Ets2 are more directly implicated as nuclear targets of Ras signal transduction pathways in mam-

malian cells, indicating that Ets family members play central roles in stable as well as transient gene expression regulated by Ras signaling pathways.

Ets1 and Ets2 are capable of collaborating with an activated Ras product to superactivate enhancers containing Ets–AP-1 composite elements or, in the case of Ets2, the E.18 Ets-Ets element. The data for the E.18 promoter indicate that Ets2 is a direct target of the Ras signaling pathway and not an auxiliary factor necessary for correct AP-1/ATF function. Although activated Ras does increase the level of expression of Ets2

from the cytomegalovirus-driven expression plasmids three- to fivefold, the synergism between the two cannot be attributed to this effect alone. First, the Ets2 alanine 72 mutation did not alter the level of protein expression but abolished reporter gene superactivation in combination with Ras. Second, the synergism is not seen with all Ets2-responsive promoters; for example, the c-*fms* promoter is Ets responsive but not Ras responsive, and the alanine 72 mutation has no effect on Ets2 activation of the c-*fms* promoter. This observation indicates that the promoter context is critical for Ras-Ets collaboration to be evident. These results also provide an important control demonstrating the integrity of the mutated Ets factors as well as the specific role of phosphorylation of the conserved threonine residues in Ras-activated signaling. Finally, Ets2 expression can be amplified independently of Ras action by increasing the amount of expression plasmid cotransfected with the reporter. Increasing Ets2 expression by three- to fivefold in this way does not lead to reporter superactivation (55).

Several other Ets factors, including Fli-1, Elf-1, and PEA3, do not collaborate with Ras in enhancer regulation. Comparison of the sequences of Ets1 and Ets2 indicated that a previously recognized homology of approximately 100 amino acids in the N-terminal regions of both proteins (52) might be responsible for the common behavior of the two factors in the assays reported here. The molecular genetic data presented support the view that phosphorylation of a potential MAP kinase site (PLLTP [1]) at position threonine 38 or threonine 72 in Ets1 or Ets2, respectively, is critical for the ability of these factors to collaborate with Ras in superactivation of RRE reporters.

Because members of the MAP kinase family can interchangeably phosphorylate either threonine or serine residues in the proper context, we tested mutants that alter Ets-2 threonine 72 to serine, which could potentially be phosphorylated, or to alanine or glutamic acid, which could not be phosphorylated. Only the Ets2 serine 72 mutant could collaborate with Ras. Additionally, biochemical evidence implicates threonine 72 of Ets2 as the sole Ras-dependent site of threonine phosphorylation in vivo. Phosphothreonine in Ets2 was detected in the presence of activated Ras but was absent in the Ets2 alanine 72 mutant coexpressed with Ras. Furthermore, our finding that the serine 72 mutant of Ets2 is fully functional in collaborating with Ras, yet contains no phosphothreonine when coexpressed with Ras, strongly suggests that residue 72 is not just a recognition sequence for the kinase but is in fact the site of Ras-induced threonine phosphorylation in wild-type Ets2 protein. These data reveal that the conserved phosphorylation site is necessary for the phenomenon of enhancer superactivation. As Ras-dependent phosphorylation of serine sites in these proteins cannot be ruled out by the data presented here, phosphopeptide mapping studies will be required to determine if serine phosphorylation of Ets2 occurs in response to Ras signaling events.

It has recently been reported that the p68 form of Ets1 can collaborate with Ras and the transcription factor PIT-1 in the activation of the prolactin promoter, and the portion of p68 Ets1 responsible for collaboration with Ras was roughly mapped within the highly conserved Pointed domain (7). However, in contrast to our results with the RRE reporters, Ets2 was incapable of activating this promoter in either the absence or presence of Ras (7). This difference may be attributed to interactions between Ets1 and PIT-1 not mimicked by Ets2 and thus be a specific example of protein-protein interactions governing whether a promoter that contains Ets factor binding sites will be Ras responsive. This hypothesis can also account for the difference in activation by Ets1 and Ets2 of the E.18

reporter used here as well as for the inability of Ets1 to activate the scavenger receptor Ets–AP-1 enhancer in F9 embryonal carcinoma cells (53). Therefore, Ras-dependent phosphorylation likely alters the interaction of Ets1 and Ets2 with other nuclear factors. This altered protein association could increase the binding affinity of Ets1 and Ets2 for a particular recognition site, increase the transactivation potential of Ets1 and Ets2 located at a particular recognition site, or affect both properties.

Differentiation of the photoreceptor R7 cell in the developing *Drosophila* eye is controlled by a Ras signaling pathway (43). The components of the fly Ras signaling pathway are remarkably conserved between flies and mammals, and the genetic analysis of this invertebrate system has been critical in unraveling many long-standing issues in the mammalian Ras field (reviewed in reference 17). Thus, analysis of the *Drosophila* factor Pointed (8, 38) provided a precedence for our studies of Ets1 and Ets2. Our data extend to mammalian cells the evidence for the importance of the Pointed domain, and in particular the conserved threonine phosphorylation site, in Ras-Ets collaboration in mammalian cells. In addition, our work advances the observations made in *Drosophila* studies in two ways. First, the use of elements from genes known to be targets of Ras signaling, instead of the artificial reporters used in the *Drosophila* work (38), highlights the critical nature of promoter context in the activation of Ets activity by Ras. Second, our data provide evidence that the crucial threonine residue is phosphorylated in response to Ras in vivo, data only inferred from genetic and in vitro studies in the case of *Drosophila* Pointed.

The sequence of the proline-flanked site and homology to the *Drosophila* Ras signaling pathway of indicate that a MAP kinase family member is likely responsible for phosphorylation of threonine 38 and threonine 72 of Ets1 and Ets2, respectively. Consistent with this idea, a glutathione S-transferase fusion protein that contained the 100 amino acid region of homology was a weak substrate (in comparison with myelin basic protein) for MAP kinase p44 in vitro (55). However, MAP kinases p42 and p44 did not synergize with Ras in the superactivation assay, and kinase-defective forms of these genes did not interfere with Ras activation of the RRE reporters (55). Thus, it is likely that the threonine 72/threonine 38 Ets kinase is a distinct member of the MAP kinase family.

At present, three different MAP kinase signaling pathways have been identified (13, 14, 16, 37). Two of these MAP kinase pathways, the Raf/MEK-1/MAPK p42/p44 and the MEKK-1/MEKK4/JNK branches, can be activated by the Ras-GTP complex (16, 37). Additionally, data indicating that Ras may alter cell morphology via activation of phosphatidylinositol 3-kinase have been presented (31). Determining the identity of the Ets kinase and the cascade leading to its activation will determine whether it represents a distinct Ras signaling pathway. The identification of a molecular target for Ras signaling in Ets2 described here will now allow the characterization of upstream members of the Ets signaling pathway. The biological relevance of the activation of Ets proteins by oncogenic Ras is indicated by the finding that dominant inhibitory Ets mutations can reverse Ras-mediated cellular transformation without blocking normal cell growth (32, 51). The identity of genes activated by this Ras/Ets pathway suggests that their altered expression may play a crucial role in neoplastic transformation (2, 5). For example, the extracellular proteases uPA and stromelysin, whose promoters contain the Ras-Ets-responsive elements described here, have been implicated in the process of tumor cell invasion and metastasis (9). Describing the molecular mechanism of activation of Ets1 and Ets2 by Ras

should provide new insights into how Ras controls cellular phenotypes distinct from cell cycle regulatory events during tumorigenesis and may provide new targets for inhibiting these biological processes.

ACKNOWLEDGMENTS

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