MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 785–791
0270-7306/93/020785-07$02.00/0

Vol. 13, No. 2

Autonomous Expression of a Noncatalytic Domain of the Focal Adhesion-Associated Protein Tyrosine Kinase pp125^{FAK}

MICHAEL D. SCHALLER, CHERYL A. BORGMAN, AND J. THOMAS PARSONS*

Department of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Received 2 September 1992/Returned for modification 8 October 1992/Accepted 3 November 1992

Integrins play a central role in cellular adhesion and anchorage of the cytoskeleton and participate in the generation of intracellular signals, including tyrosine phosphorylation. We have recently isolated a cDNA encoding a unique, focal adhesion-associated protein tyrosine kinase (FAK) that is a component of an integrin-mediated signal transduction pathway. Here we report the isolation of cDNAs encoding the C-terminal, noncatalytic domain of the *FAK* kinase, termed *FRNK* (*FAK*-related nonkinase). Both the *FAK*- and *FRNK*-encoded polypeptides, pp125^{FAK} and p41/p43^{FRNK}, are expressed in normal chicken embryo cells. pp125^{FAK} and p41/p43^{FRNK} were localized to focal adhesions, suggesting that pp125^{FAK} is directed to the focal adhesions by sequences within its C-terminal domain. We also show that the fibronectin-dependent increase in tyrosine phosphorylation of pp125^{FAK} is accompanied by a concomitant posttranslational modification of p41^{FRNK}.

The integrins are a family of heterodimeric transmembrane glycoproteins (2, 15). Their extracellular domains bind to the extracellular matrix (ECM) or to cell surface proteins (2, 15), and their short cytoplasmic domains interact with proteins of the cytoskeleton, e.g., α-actinin and talin (13, 28, 37). By virtue of this structure, the integrins can direct cell adhesion to the ECM or to other cells and concomitantly provide a linkage to proteins comprising the cytoskeleton. An increasing body of evidence suggests that integrins initiate the transduction of intracellular signals. Engagement of integrins with their respective ligands, or cross-linking with antibodies, results in changes in intracellular pH (34, 35), the release of Ca^{2+} into the cytoplasm (16, 31), and the phosphorylation of proteins on tyrosine (11, 21, 36), all of which are established responses in signalling pathways. Furthermore, integrin-dependent binding to the ECM leads to changes in differentiation programs (1, 27) and changes in gene expression (38). Thus, integrins appear to play a key role in mediating cell signalling cascades that link events at the cell surface with intracellular changes in cell structure and gene regulation.

Increases in tyrosine phosphorylation occur upon cross-linking integrins (21) and upon plating fibroblasts onto tissue culture dishes coated with fibronectin (11). Activation of platelets also induces tyrosine phosphorylation (36), and the platelet integrin gpIIb-IIIa has been shown genetically to be required for the phosphorylation of some substrates on tyrosine (6, 9). These observations suggest that there is a protein tyrosine kinase(s) that becomes activated, or an antagonistic protein tyrosine phosphatase that becomes inactivated, upon the binding of the integrins to the ECM.

We recently reported the isolation of cDNAs encoding a unique cytoplasmic protein tyrosine kinase that localizes to focal adhesions (designated FAK [focal adhesion kinase or pp125^{FAK}]) (33). The deduced primary structure of pp125^{FAK} revealed a highly conserved protein tyrosine kinase catalytic domain flanked by a large NH_2 domain, lacking SH2 and SH3 motifs, and C-terminal domains of unknown function. The localization of FAK to focal adhesions has prompted several laboratories to explore the regulation of pp125^{FAK} phosphorylation following manipulation of the integrins. The results support the hypothesis that pp125^{FAK} is an integrin-regulated protein tyrosine kinase (4, 10, 12, 20, 23).

Here, we report the isolation of additional *FAK*-related cDNAs, referred to as *FRNK* (*FAK*-related nonkinase), derived from mRNAs that encode only the C-terminal domain of pp125^{FAK}. The *FRNK*-encoded protein, p41^{FRNK}, was localized to focal adhesions, suggesting that pp125^{FAK} is directed to the focal adhesions by sequences within its C-terminal domain. In addition, we show that the fibronectin-dependent increase in tyrosine phosphorylation of pp125^{FAK} is accompanied by a concomitant posttranslational modification of p41^{FRNK}.

MATERIALS AND METHODS

Cells. Primary chicken embryo cells (CE cells) were prepared and transfected with replication-competent retroviral vectors, derived from RCAS A (14), as described previously (30). In experiments designed to examine ECM-related phenomena, cells were plated onto tissue culture dishes coated either with a solution of 0.5 mg of poly-L-lysine per ml or with fibronectin (2.5 μg/cm^2) (11).

Molecular biology. A λgt11 expression library (Clontech) and a λgt10 library (13a), containing cDNA from chicken embryos and CE cells, respectively, were screened by using the cDNA clone 10a as a probe to identify other *FAK*-related cDNAs (33). Selected cDNAs were subcloned into pBluescript, and the nucleotide sequence was determined by the method of Sanger et al. (32). The epitope-tagging vector, pctag, was created by ligating oligonucleotides encoding the 11 C-terminal residues of the large T antigen of simian virus 40 (KPPTPPPEPET), followed by two termination codons, into the multiple cloning site of pBluescript. The *FRNK*

* Corresponding author.

cDNA clone 10a and a *FAK* cDNA containing the complete open reading frame were subcloned into pctag by using a strategy that resulted in the substitution of the tag for the C-terminal 13 residues of the *FAK*- and *FRNK*-encoded proteins. For expression in vivo, these derivatives were subcloned into the replication-competent RCAS A (BP) retrovirus vector (14).

RNA analysis. RNA was isolated from CE cells by extraction with guanidine isothiocyanate followed by sedimentation through CsCl (5, 7), and mRNA was selected by using an oligo(dT) column. Samples of 7.5 μg were subjected to Northern (RNA) analysis (8, 25). DNA fragments to be used as probes were isolated from agarose gels and radiolabeled by nick translation.

Protein analysis. CE cells were lysed in modified radioimmunoprecipitation assay buffer (17), and the lysates were directly analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) or subjected to immunoprecipitation (33). Immune complexes were washed twice in modified radioimmunoprecipitation assay buffer and twice in Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) and then boiled in sample buffer, and the immunoprecipitated proteins were analyzed by Western immunoblotting (18). In some experiments, immune complexes were treated with 0.15 to 3 U of potato acid phosphatase in 100 mM MES (2-[*N*-morpholino]ethanesulfonic acid; pH 6.0) for 15 min at 37°C (3). The phosphatase in control samples was inhibited by prior addition of 100 mM sodium phosphate. The polyvalent antiserum, BC3, was generated by immunization of rabbits with TrpE-FAK fusion protein as previously described (33). This antiserum is specific for pp125^{FAK} and proteins of M_r 41,000 to 43,000. The monoclonal antibody (MAb) KT3 is directed to the C-terminal 11 residues of simian virus 40 large T antigen (24). For in vitro translation experiments, *FRNK* cDNAs were subcloned into pBluescript, and the cDNAs were transcribed in vitro by using an mCAP mRNA capping kit (Stratagene). The in vitro transcripts were used to program a rabbit reticulocyte lysate translation system (Promega, Madison, Wis.). The labeled translation products were analyzed directly by SDS-PAGE or immunoprecipitated prior to SDS-PAGE, and the radiolabeled proteins were detected by fluorography using En^{3}Hance (NEN-Dupont).

Immunofluorescence. Immunofluorescence experiments were done as described previously (30, 39), using MAb KT3, an affinity-purified goat anti-mouse secondary antibody, and a fluorescein isothiocyanate-labeled donkey anti-goat tertiary antibody.

Nucleotide sequence accession number. The sequence data shown in Fig. 1C has been deposited in the GenBank data base and has been assigned the accession number L07415.

RESULTS

During the course of experiments characterizing cDNAs encoding a novel protein tyrosine kinase, pp125^{FAK}, a candidate substrate for pp60^{src}, three other *FAK*-related yet structurally distinct cDNAs were isolated. These cDNAs represented independent isolates from two cDNA libraries and upon sequence analysis were shown to be structurally similar. The *FAK* cDNA and the newly isolated *FRNK* cDNA are illustrated schematically in Fig. 1A. The three partial *FRNK* cDNAs and the sequencing strategy applied in their analysis are shown in Fig. 1B, and the nucleotide sequence of *FRNK* is shown in Fig. 1C.

The consensus *FRNK* cDNA is 2,771 nucleotides in length, consisting of 537 nucleotides of unique 5′ sequence spliced to the 3′ 2,234 nucleotides of the *FAK* cDNA. *FRNK* contains a long open reading frame with two candidate initiation codons, separated by a single codon, near the 5′ end. The second ATG, nucleotides 585 to 587, is embedded in a sequence more favorable for translation initiation than is the first ATG, nucleotides 579 to 581; however, the precise start site for translation remains to be established. The second ATG codon corresponds to Met-668 of the predicted protein encoded by the *FAK* cDNA. The translation product of the *FRNK* cDNA is predicted to be 39,798 Da and is identical to the C-terminal 360 amino acids of the *FAK*-encoded protein. *FRNK* does not contain the nucleotide sequences encoding the catalytic domain of the *FAK* kinase.

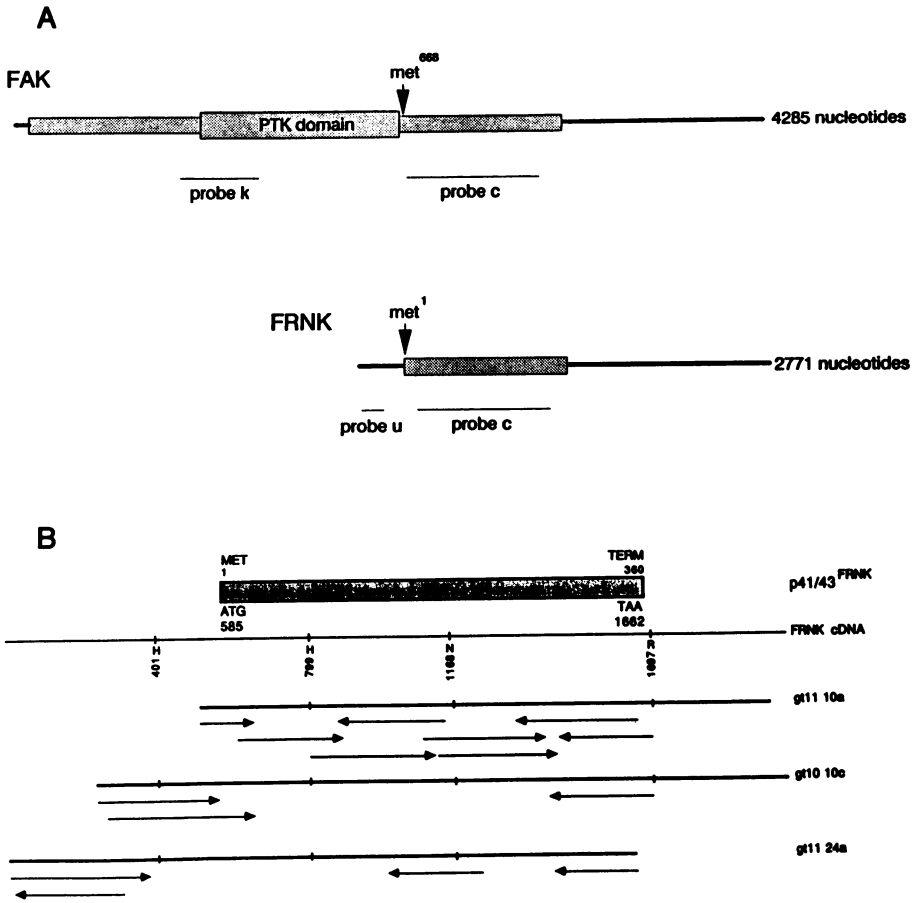
Northern blot analysis was used to confirm the expression of the *FRNK* mRNA. Poly(A)$^+$ RNA from CE cells was probed with the three different DNA fragments indicated in Fig. 1A. The fragment common to both cDNAs (probe c) hybridized to two bands, a 4.8-kb mRNA and a broad band of 2.4 to 2.8 kb which sometimes resolved into two species (Fig. 2). The fragment unique to the *FAK* cDNA (probe k) hybridized only to the 4.8-kb mRNA, and the probe unique to the *FRNK* cDNA (probe u) hybridized to the 2.8-kb mRNA. It appears that probe u hybridizes only to the upper mRNA of the 2.4/2.8-kb doublet, and thus there may be yet a third mRNA species sharing the sequences common to *FAK* and *FRNK*.

The translation products of the *FAK* and *FRNK* mRNAs were initially identified by using BC3, a polyvalent rabbit antiserum raised against a bacterially expressed fusion protein containing the C-terminal one-third of pp125^{FAK}, which includes the complete *FRNK* translation product. This antibody recognized three proteins in a direct immunoblot of CE cell lysates, pp125^{FAK} and two proteins of M_r 43,000 and 41,000 (Fig. 3). Furthermore the BC3 antibody and the anti-pp125^{FAK} MAb, 2A7, both immunoprecipitated the 43- and 41-kDa species in addition to pp125^{FAK}. Preimmune antiserum and control MAb did not react with p43 and p41. This immunological analysis suggested that one or both of these proteins was the translation product of the *FRNK* mRNA.

The *FRNK*-encoded protein was directly identified by expression of the cDNA in vitro and in vivo. *FRNK* was transcribed in vitro and translated in a reticulocyte lysate, and the product was analyzed by SDS-PAGE. The major translation product was a single protein approximately 41 kDa in size, and there were a number of minor products, one approximately 43 kDa in size and the others smaller. The minor products with a faster M_r may be generated by translation initiation at internal methionines. The proteins could be immunoprecipitated with the BC3 antiserum and MAb 2A7 but were not recognized by preimmune serum or by a control MAb (Fig. 4). From this experiment, it appeared that *FRNK* encoded a single major polypeptide, most likely the 41-kDa protein recognized by BC3. The larger minor translation product could be the BC3-reactive 43-kDa protein.

For expression in vivo, the *FRNK* cDNA was subcloned into the RCAS A (BP) retrovirus vector (14) and transfected onto cultures of CE cells. Expression of the *FRNK*-encoded protein was measured by immunoprecipitation and Western blotting using BC3. Cells infected with RCAS/*FRNK* had significantly increased levels of both p41 and p43, demonstrating that the *FRNK* cDNA encodes two proteins in vivo (Fig. 5).

One mechanism allowing a single cDNA to encode two



C

```
   1: AGACGAGGGTTAGCTGCAGCGAGACAAGATGACTTCGCTGCTTTTAAAAAGGGGCACGGATTCTTTTGGCT
  72: GGGAAAGGAAAAGCGGAGTTCTAGTCTGCAACCAAGCAGGAATTTACTCTTGAAATAAGCAATGAAGTTCCA
 144: AGATTAGAATAGATTGCTGCAGTTGATCTCATCCCTTGCATGAGTAATTCTGGGTGGTTATTTAGTAGACTG
 216: CACAGATTATAAACTCAGAGCTGAGCTCCGTTTCTCAAAAGGCTGGAGTGATAAAGAAGCTGCAATGGCTGC
 288: TTTACTGGATTGTCCCCACTCTGGAGATGGATACATAAATAATCTGCCCTCCCCAGATCTCCATGCTATCCA
 360: TGTAGTGTGCAGCTCCAGCGAATTGGCAGCTCCCTCCTCCAAAGCTTCATTCTGAGTATTTTTTAAATGGCA
 432: AATATCCAACATTAATAATCCACATAGCAGAGCTGGGAGGAGGCCTGCTGTGGGGGCAGCAGGATCACGGTG
 504: AACTTCAAACAGTCTTTTTAATAGTCAGAACTGTGTACAATACTGGAGGAGGAGAAGCTGCAGCAAGAGGAA
 576: CGAATGAGAATGGAATCCAGGCGACAAGTCACAGTATCCTGGGACTCAGGAGGATCAGATGAAGCTCCTCCC
   1:           MetGluSerArgArgGlnValThrValSerTrpAspSerGlySerAspGluAlaProPro

 648: AAGCCCAGCAGGCCTGGTTACCCCAGCCCAAGGTCCAGTGAAGGGTTTTATCCGAGTCCTCAGCATATGGTA
  22: LysProSerArgProGlyTyrProSerProArgSerSerGluGlyPheTyrProSerProGlnHisMetVal

 720: CAGCCAAATCACTACCAGGTATCTGGCTACTCTGGTTCTCATGGGATACCAGCCATGGCAGGCAGCATTTAT
  46: GlnProAsnHisTyrGlnValSerGlyTyrSerGlySerHisGlyIleProAlaMetAlaGlySerIleTyr

 792: CCTGGGCAAGCTTCTCTCTTGGATCAAACAGATTCCTGGAACCATCGACCTCAGGAAGTATCAGCATGGCAG
  70: ProGlyGlnAlaSerLeuLeuAspGlnThrAspSerTrpAsnHisArgProGlnGluValSerAlaTrpGln

 864: CCAAACATGGAGGATTCGGGCACTTTGGATGTACGAGGAATGGGGCAGGTTCTGCCCACACATCTCATGGAG
  94: ProAsnMetGluAspSerGlyThrLeuAspValArgGlyMetGlyGlnValLeuProThrHisLeuMetGlu

 936: GAGAGGTTAATAAGACAACAGCAAGAGATGGAAGAAGATCAACGCTGGCTTGAGAAAGAGGAACGATTCCTG
 118: GluArgLeuIleArgGlnGlnGlnGluMetGluGluAspGlnArgTrpLeuGluLysGluGluArgPheLeu

1008: AAACCTGATGTGCGGCTCTCCAGAGGCAGCATTGAACGGGAGGACGGAGGTCTCCAGGGCCCAGCTGGTAAC
 142: LysProAspValArgLeuSerArgGlySerIleGluArgGluAspGlyGlyLeuGlnGlyProAlaGlyAsn

1080: CAGCACATATATCAGCCTGTGGGTAAACCAGATCATGCCGCTCCACCAAAGAAGCCCCCTCGCCCTGGAGCC
 166: GlnHisIleTyrGlnProValGlyLysProAspHisAlaAlaProProLysLysProProArgProGlyAla

1152: CCCCACTTGGGCAGCCTCGCGAGCCTGAACAGCCCCGTGGACAGCTACAACGAAGGCGTGAAGATCAAGCCA
 190: ProHisLeuGlySerLeuAlaSerLeuAsnSerProValAspSerTyrAsnGluGlyValLysIleLysPro

1224: CAGGAAATCAGCCCTCCTCCTACGGCCAACCTGGACCGCTCCAATGACAAAGTCTATGAGAATGTAACCGGG
 214: GlnGluIleSerProProProThrAlaAsnLeuAspArgSerAsnAspLysValTyrGluAsnValThrGly

1296: CTGGTGAAAGCTGTCATAGAGATGTCCAGTAAAATACAGCCAGCTCCGCCAGAGGAGTACGTGCCCATGGTA
 238: LeuValLysAlaValIleGluMetSerSerLysIleGlnProAlaProProGluGluTyrValProMetVal

1368: AAGGAGGTTGGCTTGGCGCTGAGAACCTTGCTAGCAACAGTGGATGAGTCGCTGCCAGTGCTTCCTGCAAGC
 262: LysGluValGlyLeuAlaLeuArgThrLeuLeuAlaThrValAspGluSerLeuProValLeuProAlaSer

1440: ACCCACAGAGAGATTGAGATGGCCCAGAAACTGCTGAACTCTGACCTGGCTGAGCTCATTAACAAGATGAAG
 286: ThrHisArgGluIleGluMetAlaGlnLysLeuLeuAsnSerAspLeuAlaGluLeuIleAsnLysMetLys

1512: CTGGCCCAGCAGTACGTCATGACCAGCCTGCAGCAGGAGTACAAGAAGCAAATGCTGACGGCTGCTCAGGCT
 310: LeuAlaGlnGlnTyrValMetThrSerLeuGlnGlnGluTyrLysLysGlnMetLeuThrAlaAlaGlnAla

1584: CTGGCTGTGGATGCCAAGAACTTGCTGGATGTCATCGATCAAGCCAGACTGAAAATGATCAGCCAGTCCAGG
 334: LeuAlaValAspAlaLysAsnLeuLeuAspValIleAspGlnAlaArgLeuLysMetIleSerGlnSerArg

1656: CCCCACTAAGCACCGAGGGAAGGTTTCATCTGTCTGTTGTAGAATTC
 358: ProHis***
```

FIG. 1. Structures and sequences of FRNK cDNAs. (A) The *FAK* and *FRNK* cDNAs are illustrated schematically. The stippled boxes denote the open reading frame in each, and the catalytic domain of the *FAK*-encoded protein tyrosine kinase (PTK) is indicated. The position of the putative initiation codon of the *FRNK* cDNA is shown, as is the corresponding methionine codon of the *FAK* cDNA. The positions of three DNA fragments used as probes for Northern analysis are indicated. (B) Sequences of individual *FRNK* cDNAs. The arrows indicate fragments that have been sequenced. The shaded box represents the translation product from the deduced open reading frame of *FRNK*. H, *Hin*dIII; N, *Nru*I; R, *Eco*RI. (C) Nucleotide and deduced amino acid sequences of the *FRNK* cDNA. Sequences unique to *FRNK* are indicated by the bold underline. The arrow denotes the position of a two-amino-acid insertion found in a single *FAK* cDNA, presumably a polymorphism (33). A single point mutation (circled) distinguishes this cDNA from the reported sequence of the *FAK* cDNA (33) and results in the substitution of glutamine for histidine at codon 356 of the *FRNK* cDNA. The termination codon is also underlined.

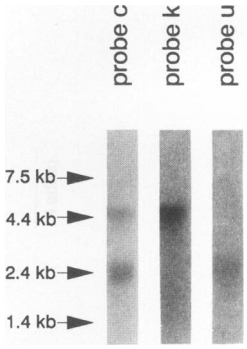


FIG. 2. Northern blot analysis of *FAK* and *FRNK* mRNAs. Poly(A)$^+$ RNA from CE cells was subjected to Northern analysis using a probe common to both the *FAK* and *FRNK* cDNAs (probe c), a probe unique to the *FAK* cDNA (probe k), and a probe unique to the *FRNK* cDNA (probe u). Arrows mark the positions of molecular size standards.

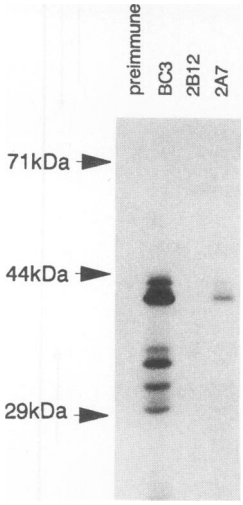


FIG. 4. Expression of the *FRNK* cDNA in vitro. The *FRNK* cDNA, subcloned into pBluescript, was transcribed and translated in vitro as described in Materials and Methods. The translation product was immunoprecipitated with MAb 2A7 (2A7) or polyclonal antiserum BC3 (BC3), both of which recognize pp125^{FAK} and the related 41/43-kDa proteins. Control immunoprecipitations using a control MAb (2B12) or preimmune rabbit serum (preimmune) were analyzed in parallel. Molecular weight markers are indicated at the left.

polypeptides is posttranslational modification of a fraction of the protein within the cell. To determine whether phosphorylation accounted for the shift in mobility of p41, a BC3 immune complex was treated with potato acid phosphatase. Phosphatase treatment reduced the p41/p43 doublet to a single polypeptide comigrating with p41 (Fig. 6). This mobility shift was blocked in the presence of a phosphatase inhibitor (Fig. 6). Thus, phosphorylation appeared to account for the difference in mobility between p41 and p43. Since p43 does not appear to contain phosphotyrosine, as determined by Western blotting with an antiphosphotyrosine antibody, nor can it be phosphorylated in vitro in pp125^{FAK}-containing immune complexes (data not shown), the sites of phosphorylation are presumably serine and/or threonine.

Since pp125^{FAK} is localized to cellular focal adhesions, the subcellular localization of p41/p43^{FRNK} was obviously of interest. However, the absence of any peptide sequences unique to p41/p43^{FRNK} precluded the generation of p41/p43^{FRNK}-specific immunological reagents. This problem was circumvented by epitope tagging the *FRNK*-encoded polypeptide as described in Material and Methods. CE cells infected with a retroviral vector expressing either the tagged

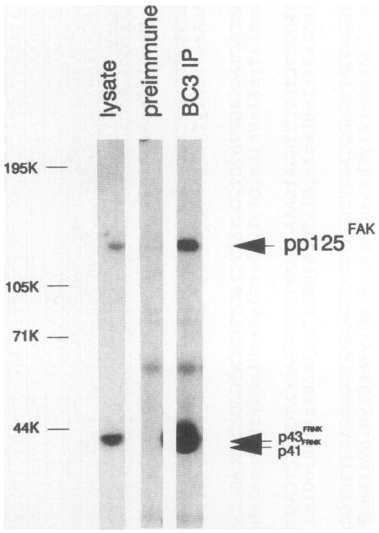


FIG. 3. Western blot analysis of *FAK*- and *FRNK*-encoded polypeptides. CE cell lysate (lysate) and the proteins immunoprecipitated from the lysate with the BC3 antiserum (BC3IP) were fractionated by SDS-PAGE and immunoblotted with the BC3 antiserum. A control immunoprecipitation using preimmune serum (preimmune) was analyzed in a similar fashion. The positions of molecular weight markers are marked at the left.

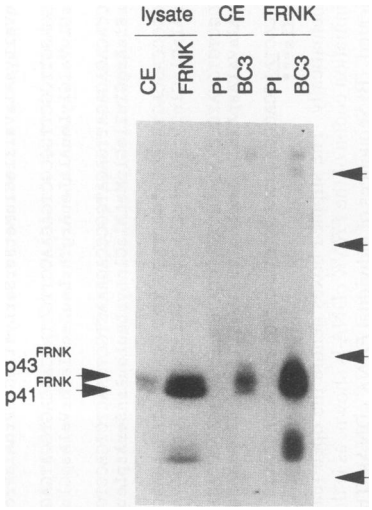


FIG. 5. Expression of the *FRNK* cDNA in CE cells. The *FRNK* cDNA was subcloned into the RCAS A (BP) retroviral vector and expressed in CE cells. Cell lysates from control cells (CE) or cells transfected with the RCAS A/*FRNK* construct (FRNK) were directly loaded onto polyacrylamide gels (lysate) or were first immunoprecipitated with the BC3 antiserum (BC3). The samples were then Western blotted with BC3 antiserum. Control immunoprecipitations using preimmune serum (PI) were carried out in parallel. Arrows (right) denote the positions of molecular weight markers (105, 71, 44, and 29 kDa).

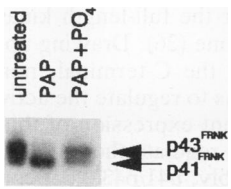


FIG. 6. Evidence that $p43^{FRNK}$ is a hyperphosphorylated variant of $p41^{FRNK}$. BC3 immune complexes containing $p43^{FRNK}$ and $p41^{FRNK}$ were incubated with buffer only (untreated), treated with 3 U of potato acid phosphatase (PAP), or treated with PAP in the presence of the inhibitor $NaPO_4$ (PAP+PO_4). The treated samples were then subjected to SDS-PAGE and Western blotted with BC3 antiserum.

$p41/p43^{FRNK}$ or, as a control, a pctag derivative of $pp125^{FAK}$ were immunostained with MAb KT3. Both $pp125^{FAK}$ (Fig. 7A) and $p41/p43^{FRNK}$ (Fig. 7B) were localized to focal adhesions, a pattern of localization identical to that previously reported for $pp125^{FAK}$. KT3 did not stain CE cells overexpressing $pp125^{FAK}$, which did not contain the tag (Fig. 7C), and CE cells expressing a tagged Myb construct produced a different pattern of immunostaining (Fig. 7D), excluding the possibility that the tag itself was responsible for the localization of these proteins within focal adhesions. These observations verified that $pp125^{FAK}$ is localized to focal adhesions in CE cells and indicates that $p41/p43^{FRNK}$ is also found localized to cellular focal adhesions.

The observation that $pp125^{FAK}$ is localized to focal adhesions identifies it as a candidate for an integrin-linked protein tyrosine kinase. Therefore, the effect of ECM proteins on $pp125^{FAK}$ was measured, using phosphotyrosine content as an indirect measure of enzymatic activity. $pp125^{FAK}$ from cells growing in culture contains phosphotyrosine. Upon trypsinization and plating on poly-L-lysine-coated tissue culture dishes, conditions under which cells adhere in an integrin-independent fashion, the phosphotyrosine content declines (Fig. 8A). In contrast, cells plated onto fibronectin, to which cells adhere via the fibronectin receptor, an integrin, exhibited a time-dependent increase in the amount of phosphotyrosine on $pp125^{FAK}$ (Fig. 8A). This observation was not due a variation in the amount of $pp125^{FAK}$ (Fig. 8A). This observation was not due to a variation in the amount of $pp125^{FAK}$. The increase in phosphorylation is likely due to autophosphorylation of $pp125^{FAK}$ (unpublished data) and suggests that the activity of $pp125^{FAK}$ is significantly increased upon fibronectin receptor binding to extracellular fibronectin.

Since $p41/p43^{FRNK}$ is also in focal adhesions and also appears to be posttranslationally modified by phosphorylation, we determined whether p41/43 phosphorylation was also induced by interactions between integrins and the extracellular matrix. In CE cells growing in culture, both $p41^{FRNK}$ and $p43^{FRNK}$ were readily detected by Western blot analysis (Fig. 8B). When cells were plated on poly-L-lysine-coated dishes, the major detectable species was $p41^{FRNK}$, suggesting that $p43^{FRNK}$ had become dephosphor-

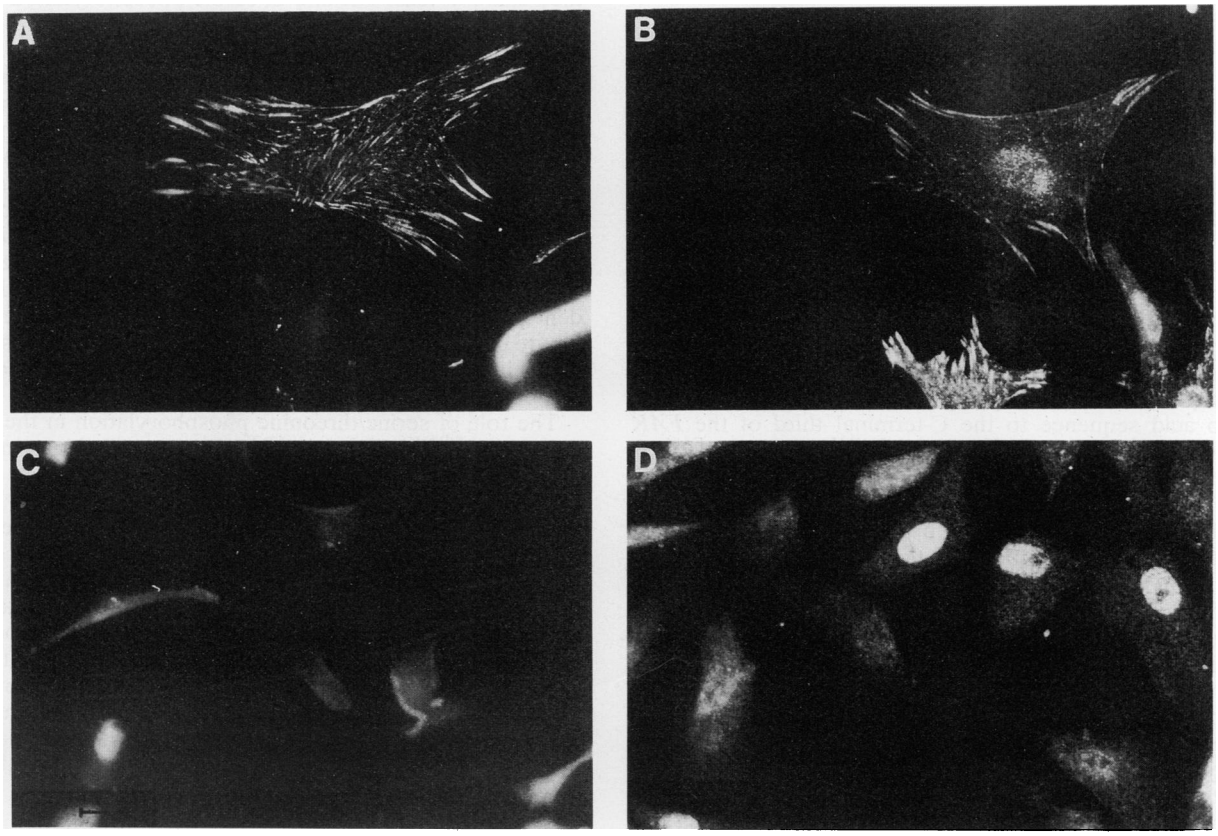


FIG. 7. Evidence that $p41/p43^{FRNK}$ is localized to focal adhesions. CE cells overexpressing epitope-tagged $pp125^{FAK}$ (A), epitope-tagged $p41/p43^{FRNK}$ (B), untagged $pp125^{FAK}$ (C), or epitope-tagged Myb (D) were immunostained with MAb KT3 as described in Materials and Methods.

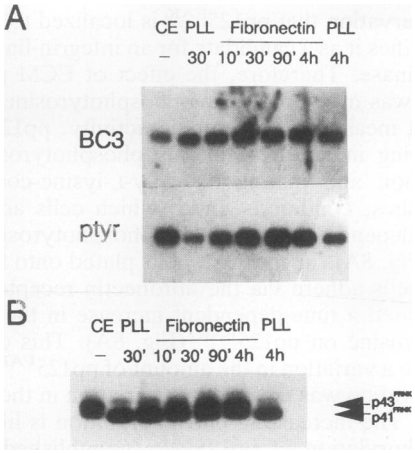


FIG. 8. Induction of pp125^{FAK} and p41^{FRNK} phosphorylation by the ECM. CE cells were plated onto dishes coated with fibronectin or poly-L-lysine (PLL) for the indicated times and were then lysed and analyzed. (A) pp125^{FAK} was isolated by immunoprecipitation with BC3 antiserum and subjected to Western blotting with either BC3 (BC3) or rabbit antiphosphotyrosine antibodies (ptyr). As a control, pp125^{FAK} was immunoprecipitated from CE cells growing in culture (CE). (B) p41^{FRNK} and p43^{FRNK} were immunoprecipitated with BC3 antiserum and detected by Western blotting with BC3.

ylated. p43^{FRNK} reappeared with time when the cells were plated onto fibronectin-coated tissue culture dishes (Fig. 8B). Therefore p41^{FRNK}, like pp125^{FAK}, becomes posttranslationally modified in an ECM-dependent manner.

DISCUSSION

The *FRNK* cDNA described here is related to the recently isolated *FAK* cDNA, which encodes a novel protein tyrosine kinase that is associated with cellular focal adhesions (33). *FRNK* is a bona fide cDNA, since the mRNA from which it was generated and its translation product, p41/p43^{FRNK}, have been identified in CE cells. While it is evident from this analysis that multiple transcripts are produced from the single gene encoding pp125^{FAK} and p41/p43^{FRNK}, the mechanism by which these mRNAs are generated is not clear. The most probable mechanisms are alternative splicing and/or utilization of alternative transcriptional promoters.

The protein encoded by the *FRNK* cDNA is identical in amino acid sequence to the C-terminal third of the *FAK* protein product, pp125^{FAK}. p41/p43^{FRNK}, like pp125^{FAK}, is localized to focal adhesions. The observation that p41/p43^{FRNK} is also targeted to focal adhesions suggests that the signal responsible for directing pp125^{FAK} to focal adhesions resides within the C-terminal third of the protein. This conclusion has been corroborated by mutational analysis of pp125^{FAK} itself (12a).

The noncatalytic domains of pp125^{FAK} are envisioned to function similarly to the noncatalytic domains of other protein tyrosine kinases, e.g., pp60^{src}, which are responsible for targeting the protein to its proper subcellular location and for mediating protein-protein interactions between the protein tyrosine kinase and regulatory proteins or substrates. It is interesting to note that several other kinases exhibit alternative splicing, resulting in the independent expression of a single noncatalytic, regulatory domain. For example, the primary transcript encoding a calmodulin-dependent protein kinase is alternatively spliced to generate mRNAs that express either the full-length kinase or the calmodulin binding domain alone (26). Drawing upon these precedents, we speculate that the C-terminal, noncatalytic domain of pp125^{FAK} functions to regulate the activity of the kinase and that the independent expression of this domain allows it to operate in *trans* to regulate the function of other proteins in the cell. Conceivably, p41/p43^{FRNK} could also function as a focal adhesion docking protein, serving to recruit other cellular proteins to the focal adhesions.

Both the *FAK*- and *FRNK*-encoded proteins become phosphorylated in an ECM-dependent manner. pp125^{FAK} becomes phosphorylated on tyrosine, and p41^{FRNK} appears to become phosphorylated on serine or threonine to generate the p43^{FRNK} isoform. Phosphopeptide analysis of pp125^{FAK} isolated from subconfluent cells labeled with $^{32}P_i$ revealed that the major sites of tyrosine phosphorylation correspond to sites on pp125^{FAK} that become autophosphorylated in vitro (33a). Therefore, the observed increase in tyrosine phosphorylation of pp125^{FAK} upon attachment of cells to fibronectin is consistent with activation of its enzymatic activity in vivo.

In addition, pp125^{FAK} may also be phosphorylated on serine/threonine in response to the attachment of cells to the ECM. Since the primary structure of p41^{FRNK} and the C-terminal domain of pp125^{FAK} are identical, the ECM-dependent phosphorylation of p41^{FRNK} may reflect a similar modification of pp125^{FAK}. This possibility is supported by the observation that there is a subtle increase in the M_r of pp125^{FAK} upon phosphatase treatment (unpublished data). Furthermore, phosphopeptide analysis of p41/43^{FRNK} and pp125^{FAK} from cells labeled with $^{32}P_i$ has demonstrated that they share at least one site of serine phosphorylation (33a). Further analysis is required to determine whether this site is phosphorylated in response to ECM attachment.

Two compelling questions arise from the analysis of *FRNK*: (i) what is the identity of the ECM-stimulated protein serine/threonine kinase responsible for phosphorylating p41^{FRNK} and possibly pp125^{FAK}, and (ii) what role does serine/threonine phosphorylation play in regulating the activity of these proteins? There are consensus phosphorylation sites for numerous protein serine/threonine kinases within the sequence of p41^{FRNK}, including candidate sites for phosphorylation by protein kinase C, cyclic AMP-dependent protein kinase, and p34^{cdc2} (19, 29). The proline-rich nature of this protein makes kinases which recognize serine/threonine flanked by prolines attractive candidates for the responsible kinase.

The role of serine/threonine phosphorylation in the function of p41^{FRNK} and pp125^{FAK} is presently unknown. Our preliminary observations suggest that serine/threonine phosphorylation does not regulate the enzymatic activity of pp125^{FAK}. Treatment of pp125^{FAK}-containing immune complexes with phosphatase does not reduce the ability of the kinase to phosphorylate an exogenous substrate in vitro (unpublished data). Therefore, we hypothesize that this phosphorylation event may regulate some other aspect of p41^{FRNK} and pp125^{FAK} activity, such as their ability to associate with other cellular proteins. This question will ultimately be solved by identifying the serine/threonine kinase(s) that phosphorylates the C-terminal domain of pp125^{FAK}, the sites which become phosphorylated, and subsequent genetic analysis of *FAK* and *FRNK*.

ACKNOWLEDGMENTS

We thank Michael Weber, Sarah Parsons, Amy Bouton, Dan Flynn, Brad Cobb, and Janice Huff, as well as Jeff Hildebrand, Tim

Jamieson, and Hong Wu, for comments and criticisms throughout the course of this work. We also thank Joe Lipsick for providing KT3 and tagged Myb.

M.D.S. is a fellow of the National Cancer Institute of Canada. This research was supported by National Institutes of Health grants CA29243 and CA40042.

REFERENCES

1. **Adams, J. C., and F. M. Watt.** 1989. Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature (London) **340**:307–309.
2. **Albelda, S. M., and C. A. Buck.** 1990. Integrins and other cell adhesion molecules. FASEB J. **4**:2868–2880.
3. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58**:1097–1105.
4. **Burridge, K., C. E. Turner, and L. H. Romer.** J. Cell Biol., in press.
5. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
6. **Ferrell, J. E., and G. S. Martin.** 1989. Tyrosine-specific protein phosphorylation is regulated by glycoprotein IIb-IIIa in platelets. Proc. Natl. Acad. Sci. USA **86**:2234–2238.
7. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**: 2633–2637.
8. **Goldberg, D. A.** 1980. Isolation and partial characterization of the Drosophila alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. USA **77**:5794–5798.
9. **Golden, A., J. S. Brugge, and S. J. Shattil.** 1990. Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. J. Cell Biol. **111**:3117–3127.
10. **Guan, J.-L., and D. Shalloway.** 1992. Regulation of pp125^{FAK} both by cellular adhesion and by oncogenic transformation. Nature (London) **358**:690–692.
11. **Guan, J.-L., J. E. Trevithick, and R. O. Hynes.** 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regul. **2**:951–964.
12. **Hanks, S. K., M. B. Calalb, M. C. Harper, and S. K. Patel.** 1992. Focal adhesion protein tyrosine kinase phosphorylated in response to cell spreading on fibronectin. Proc. Natl. Acad. Sci. USA **89**:8487–8491.

12a. **Hildebrand, J. D., M. D. Schaller, and J. T. Parsons.** Unpublished data.

13. **Horwitz, A., E. Duggan, C. Buck, M. C. Beckerle, and K. Burridge.** 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature (London) **320**:531–533.

13a. **Huff, J., and J. T. Parsons.** Unpublished data.

14. **Hughes, S. H., J. J. Greenhouse, C. J. Petropoulos, and P. Sutrave.** 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. **61**: 3004–3012.
15. **Hynes, R. O.** 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69**:11–25.
16. **Jaconi, M. E. E., J. M. Theler, W. Schlegel, R. D. Appel, S. D. Wright, and P. D. Lew.** 1991. Multiple elevations of cytosolic free Ca^{2+} in human neutrophils: initiation by adherence receptors of the integrin family. J. Cell Biol. **112**:1249–1257.
17. **Kanner, S. B., A. B. Reynolds, and J. T. Parsons.** 1989. Immunoaffinity purification of tyrosine-phosphorylated cellular proteins. J. Immunol. Methods **120**:115–124.
18. **Kanner, S. B., A. B. Reynolds, R. R. Vines, and J. T. Parsons.** 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. Proc. Natl. Acad. Sci. USA **87**:3328–3332.
19. **Kennelly, P. J., and E. G. Krebs.** 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. **266**:15555–15558.
20. **Kornberg, L. J., H. S. Earp, M. D. Schaller, J. T. Parsons, and R. L. Juliano.** J. Biol. Chem., in press.
21. **Kornberg, L. J., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano.** 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. Proc. Natl. Acad. Sci. USA **88**:8392–8396.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Lipfert, L., B. Haimovich, M. D. Schaller, B. S. Cobb, J. T. Parsons, and J. S. Brugge.** Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. J. Cell Biol., in press.
24. **MacArthur, H., and G. Walter.** 1984. Monoclonal antibodies specific for the carboxy terminus of simian virus 40 large T antigen. J. Virol. **52**:483–491.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
26. **Means, A. R., F. Cruzalegui, B. LeMagueresse, D. S. Needleman, G. R. Slaughter, and T. Ono.** 1991. A novel Ca^{2+}/calmodulin-dependent protein kinase and a male germ cell-specific calmodulin-binding protein are derived from the same gene. Mol. Cell. Biol. **11**:3960–3971.
27. **Menko, A. S., and D. Boettiger.** 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell **51**:51–57.
28. **Otey, C. A., F. M. Pavalko, and K. Burridge.** 1990. An interaction between α-actinin and the B_1 integrin subunit *in vitro*. J. Cell Biol. **111**:721–729.
29. **Pearson, R. B., and B. E. Kemp.** 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol. **200**:62–81.
30. **Reynolds, A. B., D. J. Roesel, S. B. Kanner, and J. T. Parsons.** 1989. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular *src* gene. Mol. Cell. Biol. **9**:629–638.
31. **Richter, J., J. Ng-Sikorski, I. Olsson, and T. Andersson.** 1990. Tumor necrosis factor-induced degranulation in adherent human neutrophils is dependent on CD11b/CD18-integrin-triggered oscillations of cytosolic free Ca^{2+}. Proc. Natl. Acad. Sci. USA **87**:9472–9476.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Schaller, M. D., C. A. Borgman, B. C. Cobb, A. B. Reynolds, and J. T. Parsons.** 1992. pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. USA **89**:5192–5296.

33a. **Schaller, M. D., R. R. Vines, and J. T. Parsons.** Unpublished data.

34. **Schwartz, M. A., D. E. Ingber, M. Lawrence, T. A. Springer, and C. Lechene.** 1991. Multiple integrins share the ability to induce elevation of intracellular pH. Exp. Cell Res. **195**:533–535.
35. **Schwartz, M. A., C. Lechene, and D. E. Ingber.** 1991. Insoluble fibronectin activates the Na^+/H^+ antiporter by clustering and immobilizing integrin $\alpha_5\beta_1$, independent of cell shape. Proc. Natl. Acad. Sci. USA **88**:7849–7853.
36. **Shattil, S. J., and J. S. Brugge.** 1991. Protein tyrosine phosphorylation and the adhesive functions of platelets. Curr. Opin. Cell Biol. **3**:869–879.
37. **Tapley, P., A. F. Horwitz, C. A. Buck, K. Burridge, K. Duggan, R. Hirst, and L. Rohrschneider.** 1989. Analysis of the avian fibronectin receptor (integrin) as a direct substrate for pp60$^{v\text{-}src}$. Oncogene **4**:325–333.
38. **Werb, Z., P. M. Tremble, O. Behrendtsen, E. Crowley, and C. H. Damsky.** 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. **109**:877–889.
39. **Wu, H., A. B. Reynolds, S. B. Kanner, R. R. Vines, and J. T. Parsons.** 1991. Identification and characterization of a novel cytoskeleton-associated pp60^{src} substrate. Mol. Cell. Biol. **11**: 5113–5124.