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Interaction of the E1A Oncoprotein with Yak1p, a Novel Regulator of Yeast Pseudohyphal Differentiation, and Related Mammalian Kinases

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The C-terminal portion of adenovirus E1A suppresses ras-induced metastasis and tumorigenicity in mammalian cells; however, little is known about the mechanisms by which this occurs. In the simple eukaryote *Saccharomyces cerevisiae*, Ras2p, the homolog of mammalian h-ras, regulates mitogen-activated protein kinase (MAPK) and cyclic AMP-dependent protein kinase A (cAMP/PKA) signaling pathways to control differentiation from the yeast form to the pseudohyphal form. When expressed in yeast, the C-terminal region of E1A induced pseudohyphal differentiation, and this was independent of both the MAPK and cAMP/PKA signaling pathways. Using the yeast two-hybrid system, we identified an interaction between the C-terminal region of E1A and Yak1p, a yeast dual-specificity serine/threonine protein kinase that functions as a negative regulator of growth. E1A also physically interacts with Dyrk1A and Dyrk1B, two mammalian homologs of Yak1p, and stimulates their kinase activity in vitro. We further demonstrate that Yak1p is required in yeast to mediate pseudohyphal differentiation induced by Ras2p-regulated signaling pathways. However, pseudohyphal differentiation induced by the C-terminal region of E1A is largely independent of Yak1p. These data suggest that mammalian Yak1p-related kinases may be targeted by the E1A oncogene to modulate cell growth.

INTRODUCTION

The human adenovirus early region 1A (*E1A*) gene encodes two major proteins of 289 and 243 residues, which differ only by the presence of an internal sequence of 46 amino acids unique to the larger protein (Figure 1). Comparison of the E1A sequences of a number of adenovirus serotypes has identified three regions of sequence conservation (Kimelman *et al.*, 1985; van Ormondt *et al.*, 1986), designated conserved regions 1, 2, and 3. Exon 2 of *E1A* encodes 104 amino acids that are not highly conserved between various adenovirus serotypes. Nevertheless, this C-terminal region of E1A is required for immortalization by E1A and transformation of rodent cells in cooperation with the products of the adenovirus *E1B* oncogene (Subramanian *et al.*, 1991). In contrast to its function with the E1B proteins, the C terminus of E1A acts to repress transformation in cooperation with ras and blocks the invasive and metastatic properties of ras-transformed cells (Subramanian *et al.*, 1989; Douglas *et al.*, 1991; Linder *et al.*, 1992; Boyd *et al.*, 1993). The only known protein target of the C terminus of E1A is CtBP (Schaeper *et al.*, 1995), a transcriptional corepressor (Criqui-Filipe *et al.*, 1999; Furusawa *et al.*, 1999; Meloni *et al.*, 1999; Sewalt *et al.*, 1999). Disruption of the interaction of E1A with CtBP enhances the ability of E1A to cooperate with activated ras to transform cells and increases the tumorigenic and metastatic capacity of these transformed cells. However, the interaction of CtBP with E1A may not completely account for these activities, because E1A mutants with deletions of other regions within the C terminus show similarly increased metastatic and tumorigenic properties, yet still retain the ability to interact with CtBP (Boyd *et al.*, 1993; Schaeper *et al.*, 1995).

Progress in elucidating the mechanisms by which the C-terminal region of E1A functions to modulate ras-induced transformation, tumorigenesis, and metastasis has been hampered by the technical difficulties associated with genetic analysis in mammalian cells. In contrast, powerful tools are available for molecular genetic analysis in the yeast *Saccharomyces cerevisiae*. Furthermore, expression of a foreign gene in yeast can provide an attractive means of studying gene function because many important biological processes are conserved between higher eukaryotes and yeast. Indeed,

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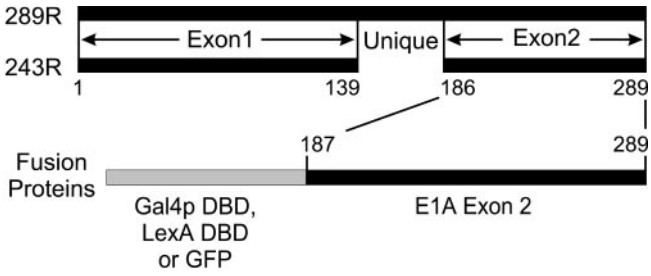

Figure 1. Map of the major E1A proteins and conserved regions. The two major products of E1A are 289 and 243 residues in length and differ only by the presence of an additional 46 amino acids unique to the larger. The C-terminal region of E1A corresponding to aa 187–289 was expressed as a fusion with the Gal4p DBD, LexA DBD, or GFP in this study.

our recent studies have established that E1A interacts with conserved gene and growth regulatory pathways in yeast (Miller *et al.*, 1995, 1996), providing a basis for more detailed investigations of the molecular mechanisms by which E1A reprograms cell growth and development.

In this study, we have examined the effects of the C-terminal domain of E1A on Ras2p-regulated growth control in *S. cerevisiae*. In this dimorphic yeast, the product of *RAS2*, the yeast homolog of mammalian *h-ras*, controls mitogen-activated protein kinase (MAPK) and cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathways to regulate pseudohyphal/filamentous differentiation in diploid cells and invasion of the growth matrix by haploid cells (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Mösch *et al.*, 1996; Cook *et al.*, 1997; Madhani *et al.*, 1997; Robertson and Fink, 1998; Pan and Heitman, 1999). Expression of the C-terminal domain of E1A induced strong pseudohyphal growth in diploid yeast, which was independent of both the MAPK and cAMP/PKA pathways and thus functions through a novel mechanism. Using the yeast two-hybrid system, we identified an interaction between the C terminus of E1A and yeast Yak1p, a dual-specificity serine/threonine kinase (Garrett *et al.*, 1991) that functions as a negative regulator of growth (Garrett and Broach, 1989).

MATERIALS AND METHODS

Yeast Strains, Media, and Microbiological Techniques

Yeast strains used in these experiments are listed in Table 1. All strains used for pseudohyphal growth assays are derived from the Σ1278b background (Grenson *et al.*, 1966). Yeast culture media was prepared and yeast genetic manipulations were performed using standard techniques (Adams *et al.*, 1998). Synthetic low-ammonia dextrose (SLAD) medium for pseudohyphal growth assay was prepared as described (Gimeno *et al.*, 1992).

Construction of Yeast and Escherichia coli *Expression Vectors*

Plasmids used in this study are listed in Table 2 and the construction of those plasmids novel to this report are summarized below. Yeast expression vector pAS1U was constructed from pAS1 (Durfee *et al.*, 1993) by subcloning the *Xba*I-*Nae*I fragment of pAS1 into the same sites of pRS426 (Christianson *et al.*, 1992). The C-terminal domain of E1A (amino acids [aa] 187–289) was expressed as a fusion with the Gal4p DBD (aa 1–147) by subcloning an *Eco*RI-*Bam*HI fragment from pMA-Ex2 (Schaeper *et al.*, 1995) into pAS1U. The C-terminal domain of E1A was expressed as fusion with the LexA DBD from pSH2-X2, which was constructed by subcloning the same *Eco*RI-*Bam*HI fragment into pSH2–1 (Hanes and Brent, 1989). The E7 protein of HPV16 was polymerase chain reaction (PCR) subcloned as an *Eco*RI-*Sal*I fragment from pATH11-E7 (Carter *et al.*, 1991) into pAS1U and pSH2–1 to make pAS1U-E7 and pSH2-E7, respectively. pRS423-LexA was constructed from pEG202 (OriGene Technologies, Rockville, MD) by subcloning the *Sph*I-*Sal*I fragment of pEG202 into the same sites of pRS423-ADH (Mumberg *et al.*, 1995). pBait was constructed by moving a *Pvu*II fragment from pRS423-LexA into the same sites of YEplac181 (Gietz and Sugino, 1988). The region encoding the C terminus of E1A was cloned as an *Eco*RI-*Bam*HI fragment from pMA-Ex2 (Schaeper *et al.*, 1995) into pBait to make pBait-X2. To construct LexA DBD fusions with E1A proteins with deletions in the C-terminal region of E1A, *Eco*RI-*Bam*HI or *Eco*RI-*Sal*I fragments from the previously described series of pMA-Ex2 deletion mutant plasmids (Schaeper *et al.*, 1995) were subcloned into corresponding sites of pBait. pGFP was constructed by subcloning an *Nhe*I-*Hin*dIII fragment of pEGFP-C1 (Clontech, Palo Alto, CA) and the self-complementary oligos JMO-44 (5′-AGCTTCT-GAATTCCCGGGGATCCCTGCAG-3′) and JMO-45 (5′-TCGACTC-CAGGGATCCCCGGGAATTCAGA-3′) into the *Spe*I-*Sal*I sites of pRS426-ADH (Mumberg *et al.*, 1995). pGFP-X2 was constructed by subcloning an *Eco*RI-*Bam*HI fragment encoding the C terminus of

Table 1. Yeast strains

Strain	Genotype	Source or Reference
JMY38a/α	*ura3-52/ura3-52 his3::hisG/his3::hisG leu2::hisG/LEU2 trp1::hisG/TRP1*	This study
L5986a/α	*ste7::LEU2/ste7::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG*	Madhani and Fink, 1997
L5987a/α	*ste12::LEU2/ste12::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG*	Liu *et al.*, 1993
L6213a/α	*phd1::hisG/phd1::hisG ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG*	Lo *et al.*, 1997
L6278a/α	*kss1::ura3::LEU2/kss1::ura3::LEU2 trp1::hisG/TRP1 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG*	Madhani and Fink, 1997
MLY61 a/α	*ura3-52/ura3-52*	Pan and Heitman, 1999
XPY1 a/α	*bcy1::G418/bcy1::G418 ura3-52/ura3-52*	Pan and Heitman, 1999
XPY5a/α	*tpk2::G418/tpk2::G418 ura3-52/ura3-52*	Pan and Heitman, 1999
XPY95a/α	*flo8::HygB/flo8::HygB ura3-52/ura3-52*	Pan and Heitman, 1999
XPY107a/α	*flo11::HygB/flo11::HygB ura3-52/ura3-52*	Pan and Heitman, 1999
DSY1a/α	*yak1::G418/yak1::G418 ura3-52/ura3-52*	Pan and Heitman, 1999
L40	*his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lexAop-LacZ) GAL4*	Invitrogen

Table 2. Plasmids

Gene	Plasmid	Description	Source or Reference
	pAS1U	*URA3* 2 μm	This study
	pBait	*LEU2* 2 μm	This study
	pGFP	*URA3* 2 μm	This study
	pSH2-1	*HIS3* 2 μm	Hanes and Brent, 1989
	pMA424	*HIS3* 2 μm	Ma and Ptashne, 1987
	pRS313	*HIS3* CEN	Sikorski and Hieter, 1989
	pRS316	*URA3* CEN	Sikorski and Hieter, 1989
	pRS423	*HIS3* 2 μm	Christianson *et al.*, 1992
	pRS426	*URA3* 2 μm	Christianson *et al.*, 1992
	YCp50	*URA3* CEN	Rose *et al.*, 1987
	YEplac195	*URA3* μm	Gietz and Sugino, 1988
	pGEX-4T-1		Amersham Pharmacia Biotech
E1A C terminus	pAS1U-X2	*URA3* 2 μm	This study
E1A C terminus	pGFP-X2	*URA3* 2 μm	This study
E1A C terminus	pMA-Ex2	*HIS3* 2 μm	Schaeper *et al.*, 1995
E1A C terminus	pSH2-X2	*HIS3* 2 μm	This study
E1A C terminus	pBait-X2	*LEU2* 2 μm	This study
E1A C terminus	pBait-X2 Δ187-221	*LEU2* 2 μm	This study
E1A C terminus	pBait-X2 Δ227-239	*LEU2* 2 μm	This study
E1A C terminus	pBait-X2 Δ239-254	*LEU2* 2 μm	This study
E1A C terminus	pBait-X2 Δ255-270	*LEU2* 2 μm	This study
E1A C terminus	pBait-X2 Δ271-284	*LEU2* 2 μm	This study
E1A C terminus	MpBait-X2 Δ285-289	*LEU2* 2 μm	This study
E1A 12S	pGST-243R		This study
E1A 12S	pRS423GAL-243R	*HIS3* 2 μm	This study
E1A 13S	pGST-289R		This study
E1A 13S	pRS423GAL-289R	*HIS3* 2 μm	This study
HPV 16 E7	pAS1U-E7	*URA3* 2 μm	This study
HPV 16 E7	pSH2-E7	*HIS3* 2 μm	This study
CtBP	pRS424-VP 16-CtBP	*TRP1* 2 μm	This study
LacZ	pIL30-URA3	*URA3* CEN	This study
LacZ	pIL30-HIS3	*HIS3* CEN	Laloux *et al.*, 1994
$RAS2^{VAL19}$	B1696	*URA3* CEN	Toda *et al.*, 1985
STE11-4	pSL 1509	*URA3* CEN	Stevenson *et al.*, 1992
STE 11-4	pRS313-STE 11	*HIS3* CEN	This study
STE7	pNC318-P368 STE7	*TRP1* CEN	Trie *et al.*, 1994
STE12	pNC252-STE12	*URA3* CEN	Errede and Ammerer, 1989
TEC1	TEC1-2mu	*URA3* 2 μm	Mösch and Fink, 1997
PHD1	pCG7	*URA3* 2 μm	Gimeno and Fink, 1994
PDE1	YEp*PDE1*	*URA3* 2 μm	Ma *et al.*, 1999
PDE2	YEp*PDE2*	*URA3* 2 μm	Ma *et al.*, 1999
BCY1	YEplac195-BCY1	*URA3* 2 μm	Pan and Heitman, 1999
TPK2	YEplac195-TPKC2	*URA3* 2 μm	Pan and Heitman, 1999
DYRK1 A	pGEX-2T-DYRK1A		Kentrup *et al.*, 1996
DYRK1 B	pGEX-2T-DYRK1B		Kentrup *et al.*, 1996
YAK1	pJG4-5YAK1	*TRP1* 2 μm	This study
YAK1	pRS426-YAK1	*URA3* 2 μm	This study

E1A from pMA-Ex2 into the same sites of pGFP. pRS313-STE11-4 was constructed by insertion of a *Bam*HI-*Sal*I fragment containing *STE11-4* from plasmid pSL1509 (Stevenson *et al.*, 1992) into pRS313 (Sikorski and Hieter, 1989). The two-hybrid prey plasmid pRS424-VP16 was constructed by subcloning a *Nhe*I-*Sal*I fragment from pVP16 (Clontech) into the *Spe*I-*Sal*I sites of pRS424-GAL1 (Mumberg *et al.*, 1995). *Drosophila* CtBP was inserted into pRS424-VP16 as a *Bam*HI fragment from plasmid *h-C28* (Poortinga *et al.*, 1998). The sequences encoding the 289R and 243R E1A proteins were PCR amplified and subcloned as *Eco*RI-*Xho*I fragments into pRS423-GAL1 to construct pRS423GAL-289R and pRS423GAL-243R, respectively (Mumberg *et al.*, 1995). The same fragments were subcloned into pGEX-4T1 (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada) to construct pGST-289R and pGST-243R. pRS426-Yak1 was constructed by PCR amplification of the *YAK1* coding region from yeast genomic DNA and subcloning it into *Bam*HI and *Xho*I sites of pRS426-ADH (Mumberg *et al.*, 1995).

Assays for Diploid Pseudohyphal/Filamentous Differentiation

Growth assays for filament formation in colonies of diploid cells were performed as described previously (Gimeno *et al.*, 1992). Briefly, diploid yeast were transformed with expression vectors by using lithium acetate (Adams *et al.*, 1998). Single colonies of transformed yeast were patched onto SLAD plates and scored for pseudohyphal filament formation after 2 d of growth at 30°C. Representative single colonies were directly photographed with a Leitz Diaplan light microscope equipped with a 40× long working dis-

tance objective and a Sony PowerHAD 3CCD color video camera using Northern Eclipse Imaging Analysis software (Empix Imaging, Mississauga Ontario, Canada).

*Assay for FG(TyA)::***LacZ** *Activity*

The plasmids pIL30-URA3 or pIL30-HIS3 (Laloux *et al.*, 1994), which contain the *LacZ* gene under the transcriptional control of a filamentation response element (Mösch *et al.*, 1996), were used to monitor MAPK-mediated activation of transcription during pseudohyphal growth. To examine the effect of the C-terminal domain of E1A on MAPK signaling, diploid yeast of the Σ1278 background were transformed with a reporter plasmid together with plasmids expressing the E1A fusion protein. β-Galactosidase assays were prepared as previously described (Mösch *et al.*, 1996). Protein concentrations from the clarified extracts were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as a standard. β-Galactosidase activity (nmol/min/mg protein) was calculated using the following equation ($OD_{420} \times 1.7$)/(0.0045 × protein concentration [mg/ml] × extract volume [ml] × time [min]) (Adams *et al.*, 1998).

Yeast Two-Hybrid Screen

Yeast strain L40 (Invitrogen, Carlsbad, CA) was transformed as described by Gietz *et al.* (1997) with pBait-X2 and a yeast genomic library in plasmid pJG4–5 (OriGene Technologies) kindly provided by Dr. M. Christman (University of Virginia, Charlottesville, VA). About 2×10^7 yeast transformants were screened for the ability to grow in the absence of histidine.

Expression of Recombinant Glutathione **S-***transferase (GST) Fusion Proteins in* **E. coli**

Vectors expressing GST fusions of either E1A, DYRK1A, or DYRK1B (Kentrup et al., 1996) were introduced into BL21 *E. coli* cells and recombinant fusion proteins were prepared and purified using glutathione-Sepharose as described by the manufacturer (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada).

Preparation of Yeast Cell Extracts and Coprecipitation Testing

Yeast cells transformed with pRS423GAL-289R and pRS423GAL-243R, which express the larger or smaller major E1A protein under the control of the *GAL1* promoter, were grown in synthetic complete selection medium containing glucose until the cell density reached 0.8. Cells were pelleted, washed with water, and resuspended in selection medium containing galactose for 6 h. Cells were collected by centrifugation, resuspended in 100 mM Tris-HCl buffer (pH7.5) containing 1 mM dithiothreitol and 20% glycerol, and disrupted with glass beads by vortexing. Glass beads and cell debris were removed by centrifugation at 4°C. The supernatant was used for in vitro interaction assays with *E. coli*-produced GST-DYRK fusions. Purified GST-DYRK fusion proteins were incubated with yeast extracts containing E1A overnight at 4°C in phosphate-buffered saline buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for protein complex formation. GST-DYRK protein complexes were purified by affinity absorption to glutathione-Sepharose by using standard procedures, separated by SDS-PAGE, and E1A was detected by Western blotting with the E1A monoclonal antibody M73 (Harlow *et al.*, 1985).

Assays of Protein Kinase Activity

To detect the effect of E1A on DYRK phosphorylation activity, GST-DYRK proteins were incubated with GST-E1A fusion protein in phosphorylation buffer (33 mM HEPES, 6.6 mM manganese chloride, 6.6 mM magnesium chloride, 0.7 mM dithiothreitol) containing 0.66 μg/μl histone H3 (Sigma-Aldrich Canada, Oakville, Ontario, Canada) for 2 h at 4°C. Reactions were started by introduction of 2 μCi of [^{32}P]ATP (Amersham Pharmacia Biotech) and were carried out at 30°C for 30 min. Reactions were terminated by the addition of 2× sample loading buffer and boiling. Samples were resolved on 15% SDS-PAGE gels, which were then dried and subjected to analysis using a Molecular Dynamics phosphorImager (Sunnyvale, CA).

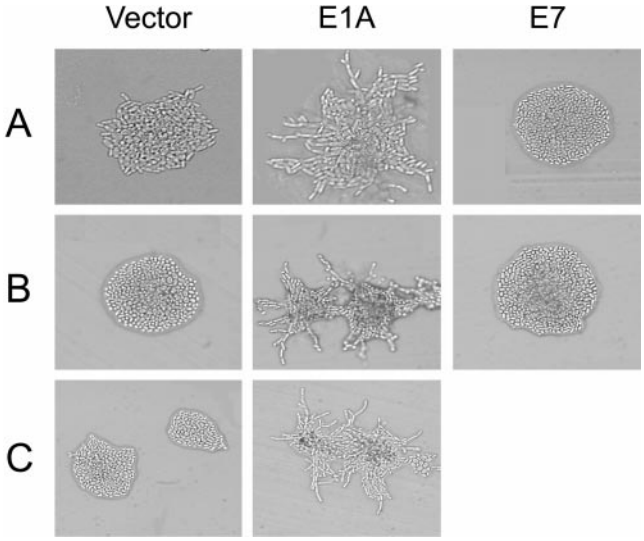

Figure 2. Effect of the C-terminal domain of E1A on yeast pseudohyphal growth. Wild-type diploid yeast of the Σ1278b background (JMY38 a/α) were transformed with a control vector; with vectors expressing the C-terminal region of E1A fused to the Gal4p DBD (A), LexA DBD (B), or GFP (C); or with vectors expressing the HPV 16 E7 protein similarly fused to the Gal4p (A) or LexA DBD (B). Transformed yeast were grown on SLAD medium for 2 d at 30°C and photographed.

RESULTS

C-Terminal Domain of E1A Enhances Yeast Pseudohyphal Differentiation

Under conditions of nitrogen starvation, diploid yeast of the Σ1278b background elongate, begin to bud in a unipolar manner, and form chains of cells in a process that has been referred to as pseudohyphal differentiation (Gimeno *et al.*, 1992). Pseudohyphal differentiation is regulated by the *RAS2* product via MAPK and cAMP/PKA signaling pathways (see INTRODUCTION). We reasoned that the C terminus of E1A might affect Ras2p function in yeast cells as it does ras function in mammalian cells, and examined whether expression of E1A in yeast affected diploid pseudohyphal growth. Expression of the C terminus (residues 187–289) of E1A fused to the yeast Gal4p DNA binding domain (DBD), the prokaryotic LexA DBD, or green fluorescent protein (GFP) (Figure 1) strongly enhanced yeast pseudohyphal growth compared with yeast transformed with the parent vectors (Figure 2). This effect appeared specific to E1A, as otherwise identical vectors expressing the comparably sized human papilloma virus (HPV) 16 E7 protein fused to the Gal4p or LexA DBD had no effect on pseudohyphal growth (Figure

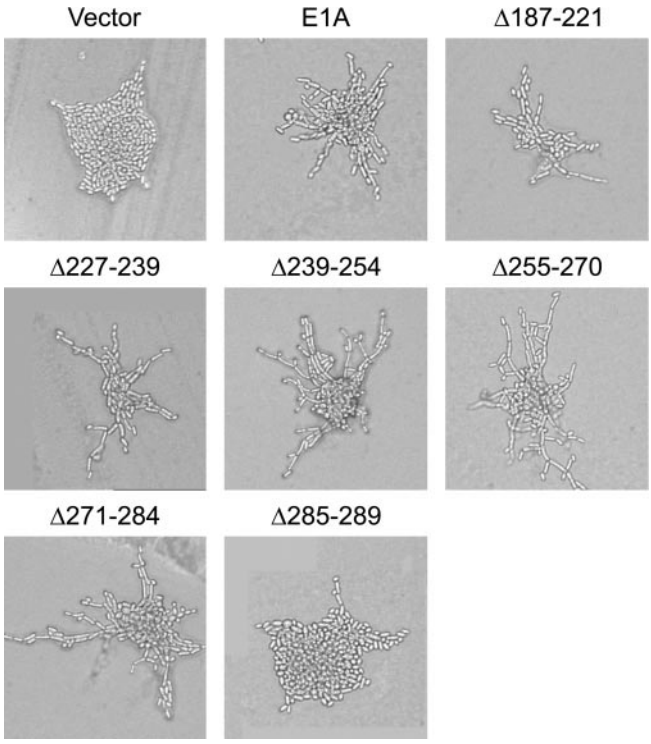

Figure 3. Induction of pseudohyphal growth by E1A mutants containing small deletions in the C-terminal region. Wild-type diploid yeast of the Σ1278b background (JMY38 a/α) were transformed with a control vector, a vector expressing the C-terminal region of E1A fused to the Gal4p DBD, or similar vectors expressing mutant forms of the C-terminal region of E1A containing the indicated amino acid deletions. Transformed yeast were grown on SLAD medium for 2 d at 30°C and photographed.

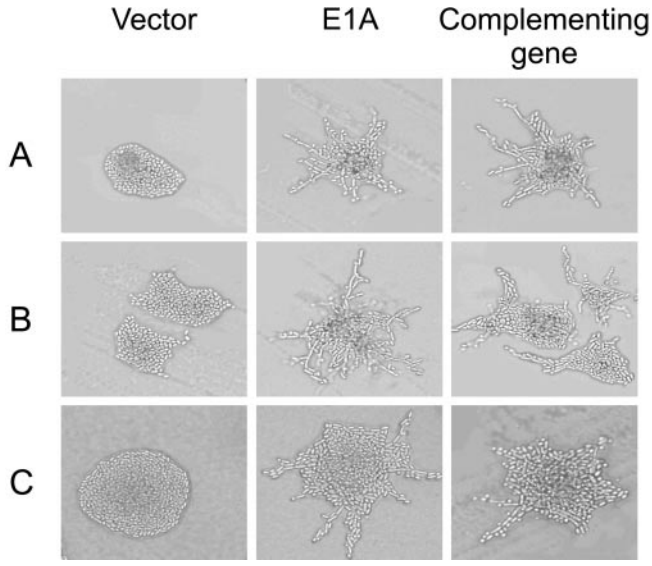

Figure 4. Ability of the C-terminal domain of E1A to induce pseudohyphal growth in yeast mutated for components of the MAPK signal transduction pathway. Diploid yeast strains with homozygous disruptions of *STE7* (L5986) (A), *KSS1* (L6278) (B), or *STE12* (L5987) (C) were transformed with either a control vector without E1A (left column) or a vector expressing the C-terminal region of E1A (middle column). Transformants were grown on SLAD medium for 2 d at 30°C and photographed. Diploid yeast of the same strains transformed only with complementing vectors expressing *STE7* (A), *KSS1* (B), or *STE12* (C) are shown in right column.

2). Enhancement of pseudohyphal growth by E1A was observed on low-nitrogen medium but not on rich medium (our unpublished results). We also assessed the ability of a series of deletion mutants within the C-terminal domain of E1A to induce pseudohyphal growth (Figure 3). The region of E1A required for induction of pseudohyphal growth was mapped to aa 284–289, the last five residues of E1A.

Independence of the E1A Effect from the MAPK Signal Transduction Pathway

The MAP kinase signaling cascade consists of Ste20p, Ste11p, Ste7p, Kss1p, and the transcription factor Ste12p, which functions together with another transcription factor, Tec1p, to activate expression of genes required for pseudohyphal growth (Madhani and Fink, 1998). To test whether the C-terminal domain of E1A enhanced pseudohyphal differentiation through this MAPK cascade, we expressed the C-terminal region of E1A in diploid strains that are homozygous for the disruption of genes encoding components of this pathway. Disruption of *STE7*, *KSS1*, or *STE12* abolished pseudohyphal growth in yeast that were not expressing E1A (Figure 4). However, expression of the C-terminal region of E1A strongly induced pseudohyphal growth in these mutant strains (Figure 4), indicating that this domain of E1A enhanced pseudohyphal growth independently of this yeast MAPK signal transduction pathway.

To test this conclusion further, we used an FG(TyA)::*LacZ* reporter construct, which is activated by Ste12p and Tec1p in response to MAPK activation under nitrogen starvation conditions (Madhani and Fink, 1997). In diploid wild-type yeast, this reporter was strongly stimulated by expression of the constitutively active *STE11-4* allele. However, transcription from this reporter was not induced by expression of the C-terminal region of E1A (Table 3). Moreover, expression of the C-terminal region of E1A did not induce reporter gene transcription in homozygous *ste12*Δ diploids (Table 3), although it enhanced pseudohyphal growth in this strain (Figure 4C). Thus, the biochemical analyses support the conclusion from the genetic studies that the C-terminal domain of E1A stimulates pseudohyphal growth independently of the MAPK cascade.

Independence of the E1A Effect from the cAMP/PKA Signal Transduction Pathway

Ras2p also stimulates pseudohyphal growth via the cAMP/PKA pathway. In particular, Ras2p stimulates adenylate cyclase activity, and the resultant accumulation of cAMP activates Tpk2p, one of the three yeast isoforms of the catalytic subunit of PKA (Robertson and Fink, 1998). Tpk2p phosphorylates the transcription factor Flo8p, which is involved in the activation of transcription from genes required for pseudohyphal growth, including *FLO11* (Pan and Heit-

Table 3. Effect of the C-terminal portion of E1A on expression of the FG(TyA)::*LacZ* reporter

Strain	Relevant genotype	Plasmid	β-Galactosidase activity* (nmol/(min × mg))
JMY38a/α	WT	Vector	15 ± 4.6
		C terminus of E1A	19 ± 10
		STE11-4	110 ± 54
L5987a/α	*ste12*E/*ste12*E	Vector	4.5 ± 1.6
		C terminus of E1A	1.9 ± 1.0
		STE12	32 ± 6.3

* Average of three independent experiments with standard deviation.

man, 1999). To test if the C-terminal domain of E1A enhanced pseudohyphal differentiation through the cAMP/PKA signal transduction pathway, we first expressed the C-terminal region of E1A in diploid strains overexpressing genes that negatively regulate this pathway. To determine whether induction of pseudohyphal growth by the C-terminal region of E1A is dependent on increased levels of cAMP, we tested the effect of overexpressing *PDE1* or *PDE2*, which encode low- and high-affinity phosphodiesterases, respectively (Ma *et al.*, 1999). Although overexpression of either *PDE1* or *PDE2* inhibited pseudohyphal growth in yeast that were not expressing E1A, this had no effect on the ability of E1A to induce pseudohyphal growth (Figure 5, A and B). Similarly, overexpression of *BCY1*, which encodes the negative regulatory subunit of PKA (Toda *et al.*, 1987), abolished pseudohyphal growth in yeast that were not expressing E1A, but did not affect enhancement of pseudohyphal growth by the C-terminal domain of E1A (Figure 5C). Thus, pseudohyphal growth by E1A does not require increased levels of cAMP or enhancement of PKA signaling.

We further tested whether the C-terminal region of E1A functioned via the cAMP/PKA signal transduction pathway by disrupting components of this cascade and examining whether this would block the enhancement of pseudohyphal growth conferred by E1A. We expressed the C-terminal domain of E1A in diploid strains homozygous for disruptions in *TPK2*, *FLO8*, or *FLO11*. Each of these disruptions abolished pseudohyphal growth in yeast that were not expressing E1A, but had no effect on the ability of E1A to induce pseudohyphal growth (Figure 5, D–F), strongly supporting the conclusion that the C-terminal domain of E1A enhances pseudohyphal growth independently of the cAMP/PKA signaling pathway.

E1A Requires Phd1p for Function

PHD1 encodes a putative transcription factor that enhances pseudohyphal growth independently of the MAPK and cAMP pathways (Chandarlapaty and Errede, 1998; Pan and Heitman, 2000). Because the C-terminal region of E1A also functions independently of the MAPK and cAMP pathways, we asked whether it depends on Phd1p for function. Consistent with previous reports, the *phd1*Δ strain formed pseudohyphae poorly (Lo *et al.*, 1997) and this was fully complemented by introduction of *PHD1* (Figure 6). Expression of the C-terminal domain of E1A failed to enhance pseudohyphal growth in this strain, indicating that it requires Phd1p for function.

Interaction of Yeast Yak1p with the C-Terminal Domain of E1A

To attempt to identify proteins with which the C-terminal domain of E1A interacts to stimulate pseudohyphal growth, we used the yeast two-hybrid screen. L40 yeast cells were transformed with a bait plasmid expressing the C-terminal region of E1A fused to the DNA binding domain of LexA and a library of yeast genomic DNA fragments fused to a transcriptional activation domain. We isolated one positive clone encoding a C-terminal fragment (aa 163–807) of the yeast dual specificity serine/threonine protein kinase Yak1p. Yak1p interacted specifically with the C-terminal domain of E1A and not with conserved region 2 of E1A or with comparably sized fragments of mouse CBP or human SUG1 fused to LexA (our unpublished results). Interestingly, Yak1p was originally identified as a negative regulator of cell growth that functions in opposition to the RAS-regulated cAMP/PKA pathway (Garrett and Broach, 1989; Ward and Garrett, 1994), but little is known about Yak1p function in yeast pseudohyphal signaling.

To identify the regions within the C-terminal domain of E1A that are required for interaction with Yak1p, two-hybrid interaction studies were performed with plasmids expressing a series of deletion mutants within the C-terminal region of E1A fused to the LexA binding domain. In addition to Yak1p, we used CtBP, the only other cellular protein known to interact with the C-terminal domain of E1A, as a control. The region of the C-terminal domain required for interaction with *Drosophila* CtBP was mapped to aa 271–284, which contain the CtBP binding motif (PLDLS), and aa 239–254 (Figure 6). This is consistent with a previous report (Boyd *et al.*, 1993), although the mutant Δ239–254 retained a measurable, but weaker interaction with human CtBP. This difference may be related to species differences between human and *Drosophila* CtBP or differences in the fusion constructs. Using the same deletion mutants, we determined that aa 187–221 and aa 239–284 of the C-terminal domain of E1A are required for interaction with Yak1p (Figure 7).

Interaction of E1A with Mammalian Homologs of Yak1p

Yak1p-related proteins represent a novel subfamily of protein kinases with unique structural and enzymatic features, which have been categorized as the dual-specificity, Yak-related kinases (Dyrks) (Becker and Joost, 1999). The catalytic domain of yeast Yak1p shares the highest homology

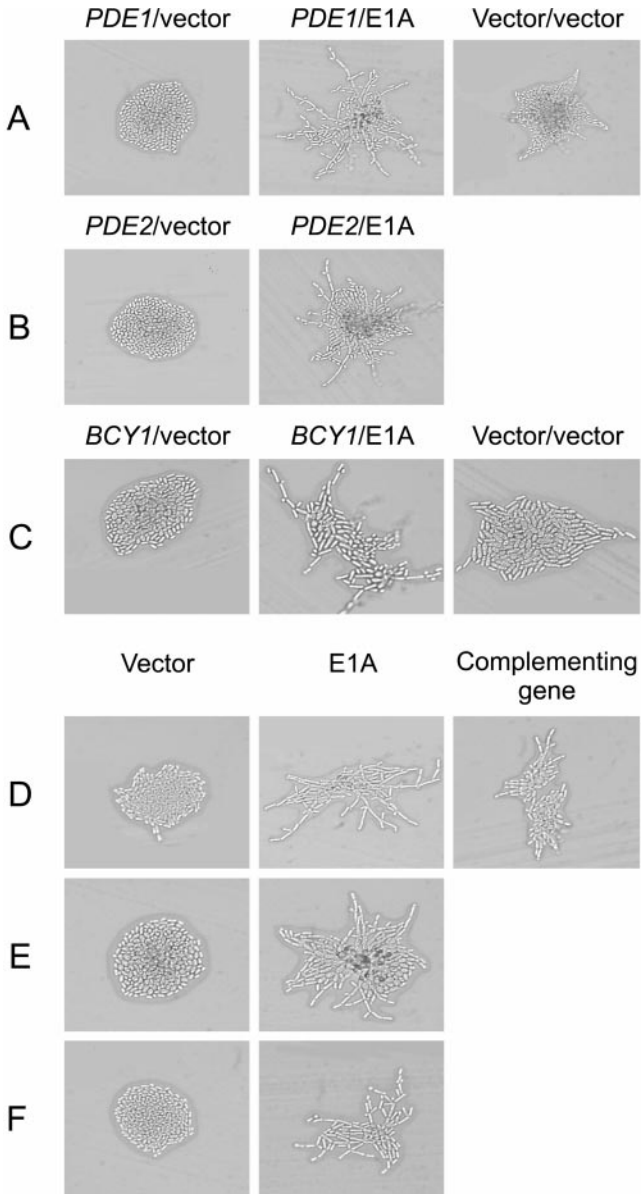

Figure 5. Ability of the C-terminal domain of E1A to induce pseudohyphal growth in yeast with alterations in the cAMP/PKA signal transduction pathway. The indicated transformants were grown on SLAD medium for 2 d at 30°C and photographed (A–E). Wild-type diploid yeast (JMY38 a/α) were transformed with vectors overexpressing *PDE1* (A), *PDE2* (B), or *BCY1* (C) and either a control vector without E1A (left column) or a vector expressing the C-terminal region of E1A (middle column). Yeast of the same strain transformed with two empty control vectors are shown in the right column. (D–F) Diploid yeast strains with homozygous disruptions of *TPK2* (XPY5) (D), *FLO8* (XPY95) (E), or *FLO11* (XPY107) (F) were transformed with either a control vector without E1A (left column) or a vector expressing the C-terminal region of E1A (middle column). (D) Cells of strain XPY95 transformed with a vector expressing *TPK2* are shown in the right column.

with the mammalian Dyrk1 proteins (Kentrup *et al.*, 1996). To ask whether E1A might physically interact with the Dyrks, we conducted in vitro protein binding assays by

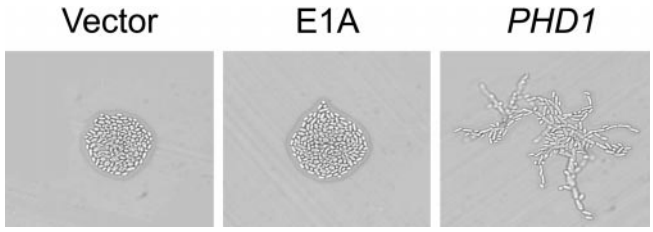

Figure 6. Dependence of the E1A effect on Phd1p. A diploid yeast strain homozygously disrupted for *PHD1* (L6213) was transformed with a control vector without E1A, a vector expressing the C-terminal region of E1A, or a vector expressing *PHD1*. Transformants were grown on SLAD medium for 2 d at 30°C and photographed.

using the rat *DYRK1A* and human *DYRK1B* products expressed as GST fusion proteins in *E. coli* (Figure 8). Both the 243R and 289R E1A proteins were efficiently coprecipitated with the recombinant GST-Dyrk1A and GST-Dyrk1B, but not with GST alone, suggesting that mammalian Dyrk1A and Dyrk1B proteins physically interact with E1A.

An unusual enzymatic property of Yak1p-related kinases is their ability to catalyze tyrosine-directed autophosphory-

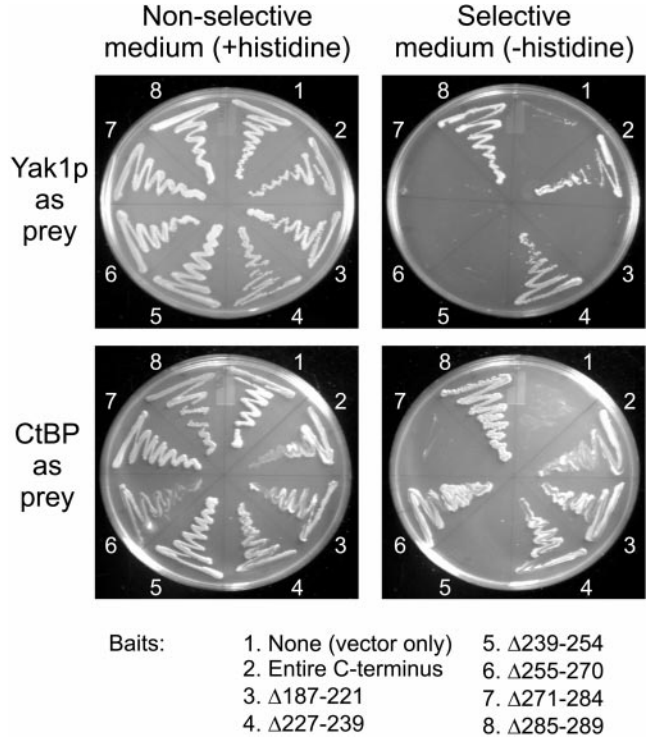

Figure 7. Interaction of yeast Yak1p with the E1A C-terminal deletion mutants in the yeast two-hybrid assay. Yeast strain L40 was transformed with expression vectors for the indicated LexA-E1A fusions and expression vectors for *YAK1* or CtBP fused to a transcriptional activation domain. Transformed yeast were streaked on nonselective plates (+histidine) and selection plates (−histidine) and allowed to grow at 30°C for 3 d. For each E1A mutant used as "bait," the indicated numbers are inclusive and refer to the amino acid residues deleted with respect to the 289R E1A protein.

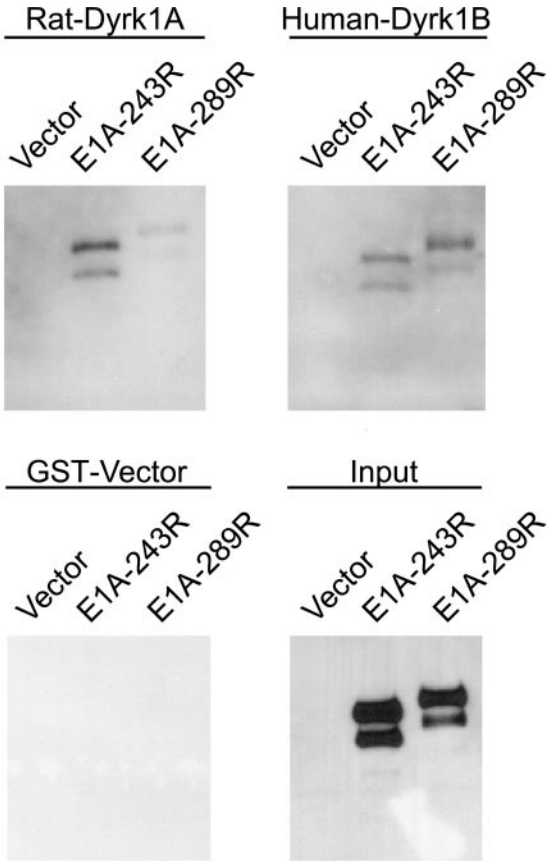

Figure 8. Coprecipitation of E1A with mammalian homologs of Yak1p. Purified recombinant rat GST-Dyrk1A or human GST-Dyrk1B was incubated with yeast extracts containing either the 243R or 289R E1A proteins (see MATERIALS AND METHODS). The GST-Dyrk protein complexes were then pulled down with glutathione-Sepharose beads and the proteins were analyzed by SDS-PAGE and imunoblotted with a monoclonal antibody against E1A. The empty GST vector was used as a negative control, and the input levels of E1A were as shown.

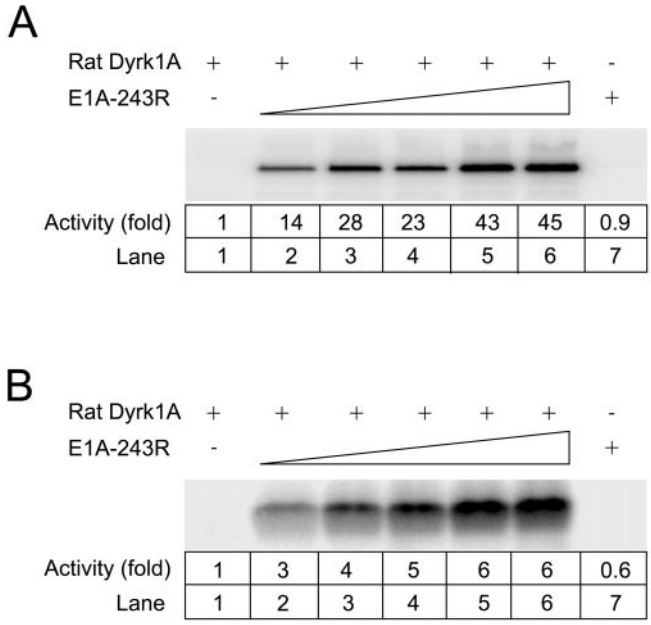

Figure 9. Effect of E1A on kinase activity of recombinant rat Dyrk1A. (A) Effect of E1A on autophosphorylation. GST-Dyrk1A was incubated with GST-E1A 243R in phosphorylation buffer for 2 h on ice. Following the addition of [γ-^{32}P]ATP, samples were incubated for 30 min at 30°C, resolved on 15% SDS-PAGE, and analyzed using a Molecular Dynamics phosphoImager. Lane 1 contains 12 μg of GST-Dyrk1A. Lanes 2–6 contain 12 μg of GST-Dyrk1A with 2, 4, 6, 8, or 12 μg of GST-E1A 243R. Lane 7 contains 12 μg of GST-E1A 243R. (B) Effect of E1A on phosphorylation of histone H3. The assay was performed as described in A except for the presence of histone H3. Fold changes in kinase activity are indicated below each lane.

lation, as well as the phosphorylation of serine/threonine residues in exogenous substrates (Becker and Joost, 1999). To define the biochemical consequences of the interaction of E1A with mammalian Dyrks, we examined the effect of E1A on recombinant rat Dyrk1A kinase activity in vitro. Recombinant GST-E1A significantly enhanced the ability of rat GST-Dyrk1A to phosphorylate itself (Figure 9A) and histone H3 (Figure 9B) as substrate in a dose-responsive manner. No effect on Dyrk1A activity was observed upon addition of GST alone (our unpublished results). Thus, the interaction between Dyrk1A and E1A stimulated its kinase activity.

Regulation of Pseudohyphal Growth by Yak1p

Overexpression of *YAK1* in wild-type diploid yeast induced strong pseudohyphal growth compared with yeast transformed with a control vector (Figure 10). However, a previous study had reported that *YAK1* is not essential for pseudohyphal differentiation, because diploid yeast with homozygous deletions in *YAK1* were still able to undergo pseudohyphal growth (Pan and Heitman, 1999). We also tested the ability of various regulators of pseudohyphal growth to induce filamentation in diploid *yak1*Δ yeast. Expression of the constitutively active *RAS2*^{VAL19}, of components of the MAPK cascade (*STE11–4*, *STE12*, or *TEC1*), or of

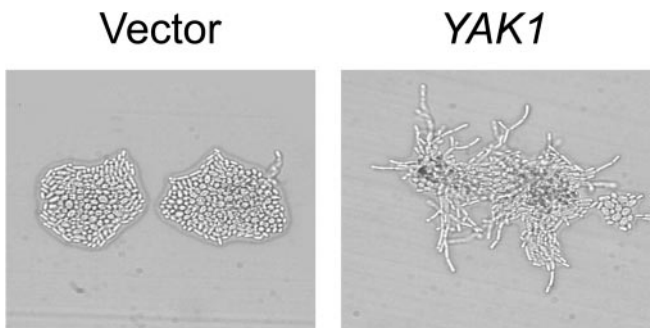

Figure 10. Effect of Yak1p overexpression on yeast pseudohyphal growth. Wild-type diploid yeast (JMY38 a/α) were transformed with a control vector or a vector expressing full-length *YAK1* under the transcriptional control of the *ADH1* promoter. Transformed yeast were grown on SLAD medium for 2 d at 30°C and photographed.

TPK2 (the central component of the cAMP/PKA pathway) all induced strong pseudohyphal growth in a wild-type strain, but not in the *yak1*Δ mutant (Figure 11), indicating that Yak1p is essential for Ras2p regulation of pseudohyphal growth through both the MAPK and cAMP/PKA pathways. In contrast, overexpression of *PHD1* induced strong pseudohyphal growth in both the wild-type and *yak1*Δ strain. Similarly, expression of the C terminus of E1A induced pseudohyphal growth in both the wild-type and *yak1*Δ mutant strains, although to a somewhat lesser extent in the *yak1*Δ mutant strain. These results are consistent with a role for Yak1p in modulating both the MAPK and cAMP/PKA signaling pathways that control pseudohyphal differentiation.

DISCUSSION

The E1A proteins of adenovirus have opposing effects on the functions of the mammalian *ras* oncogene product (Mymryk, 1996). Although E1A cooperates with activated ras to oncogenically transform cells (Ruley, 1983), it also suppresses ras-induced metastasis and tumorigenicity (Subramanian *et al.*, 1989; Douglas *et al.*, 1991; Linder *et al.*, 1992; Boyd *et al.*, 1993). Little is known about the mechanisms by which E1A modulates ras function in mammalian cells. The development of a simple model system in which the interactions between E1A and ras could be analyzed genetically would facilitate the elucidation of these interactions and their attendant regulatory pathways. Because extensive experimentation has shown that many regulatory mechanisms are conserved between the simple eukaryote *S. cerevisiae* and higher eukaryotic cells, we decided to investigate the effects of E1A on Ras2p function in this budding yeast.

In this study, we demonstrated that the C-terminal domain of adenovirus E1A strongly enhanced yeast pseudohyphal growth (Figure 2) and this requires the last five residues of E1A (Figure 3). E1A functions independently of the Ras2p-regulated MAPK and cAMP/PKA pathways to enhance pseudohyphal growth (Figures 4 and 5), suggesting that it functions either downstream of these pathways or via a third parallel regulatory pathway. This is further supported by our observation that the induction of pseudohyphal growth by the C-terminal region of E1A requires Phd1p (Figure 6), an enhancer of pseudohyphal growth that can function independently of the MAPK and cAMP/PKA pathways (Chandarlapaty and Errede, 1998; Pan and Heitman, 2000).

Using a yeast two-hybrid interaction screen to identify proteins that interact with the C terminus of E1A, we isolated a clone encoding aa 163–807 of the yeast Yak1p protein. Yak1p is a protein kinase of 807 amino acids that functions as a negative regulator of the Ras2p-regulated cAMP/PKA signal transduction pathway (Garrett and Broach, 1989). The cAMP/PKA signal transduction pathway is essential for the progression of yeast cells through the G0/G1 transition of the cell cycle (Garrett and Broach, 1989; Ward and Garrett, 1994). Yeast Yak1p was originally identified in a screen for mutants that suppress the growth defect in *RAS* mutant strains (Garrett and Broach, 1989). *YAK1* mutation also restores growth in a strain lacking the three redundant PKA catalytic subunit genes (Ward and Garrett, 1994). Thus, Yak1p appears to act as an antagonist of the Ras2p-cAMP/PKA pathway and as a negative regulator of

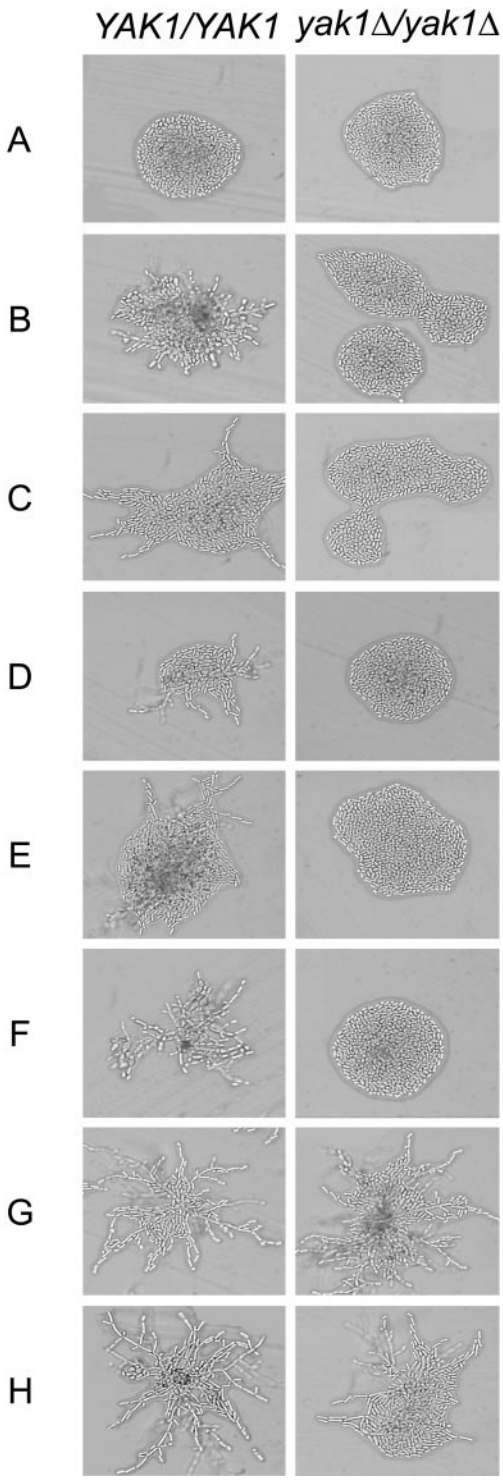

Figure 11. Genetic analysis of the role of Yak1p in regulating pseudohyphal growth. Wild-type diploid yeast (MLY61 a/α) or yeast disrupted for *YAK1* (DSY1 a/α) were transformed with a control vector (A), or with vectors expressing $RAS2^{VAL19}$ (B), *STE11-4* (C), *STE12* (D), *TEC1* (E), *TPK2* (F), *PHD1* (G), or the C terminus of E1A (H). Transformed cells were then transferred to SLAD plates, allowed to grow for 2 d at 30°C, and photographed.

growth. However, Yak1p arrests growth only in yeast strains that are attenuated in the Ras2p-cAMP/PKA pathway and overexpression has no apparent effects on otherwise wild-type yeast cells (Garrett *et al.*, 1991).

We performed a number of tests to determine whether Yak1p plays a role in regulating the signal transduction pathways that control pseudohyphal growth. Overexpression of *YAK1* induced strong pseudohyphal growth in diploid yeast cells (Figure 10). In addition, disruption of both copies of *YAK1* in diploid yeast had profound effects on the ability of Ras2p, MAPK or cAMP/PKA components to stimulate pseudohyphal differentiation (Figure 11). A role for Yak1p in pseudohyphal growth is consistent with previous work showing that Yak1p kinase activity is stimulated by nitrogen starvation (Garrett *et al.*, 1991), the same signal used to stimulate pseudohyphal growth in these studies. Although our studies provide strong evidence that the Yak1p kinase modulates both the RAS-dependent MAPK and cAMP/PKA pathways, the exact effect on RAS-regulated signal transduction remains to be addressed. The recent observation that Yak1p interacts with the Hrt1p component of the Skp1p-Cdc53p-F-box complex (Uetz *et al.*, 2000) may provide insight into the mechanism by which Yak1p affects pseudohyphal growth. The Skp1p-Cdc53p-F-box complex normally ubiquitinates the G1 cyclins Cln1p and Cln2p, signaling their proteolytic destruction (Skowyra *et al.*, 1999). Interference with this process by Yak1p might stabilize Cln1p or Cln2p, both of which are essential for pseudohyphal growth, and each of which can promote cell elongation when overexpressed (Loeb *et al.*, 1999). Alternatively, overexpression of Yak1p has also been shown to suppress the growth defects in late mitotic mutants, which characteristically exhibit increased levels of the G2/M cyclin Clb2p (Jaspersen *et al.*, 1998). Interestingly, disruption of *CLB2* induces constitutive pseudohyphal growth and overexpression of *CLB2* can inhibit pseudohyphal growth (Ahn *et al.*, 1999), suggesting that Yak1p could enhance pseudohyphal growth by reducing Clb2p expression or antagonizing Clb2p function.

Disruption of *YAK1* had no effect on the ability of Phd1p and little effect on the ability of the C terminus of E1A to stimulate pseudohyphal growth (Figure 11). These results are consistent with previous observations that Phd1p functions independently of the MAPK and cAMP/PKA pathways to induce pseudohyphal growth (Chandarlapaty and Errede, 1998). Importantly, although the C-terminal region of E1A binds to Yak1p, this appears to mediate only a small portion of the ability of E1A to induce pseudohyphal differentiation. This is consistent with our genetic (Figures 4 and 5) and biochemical data (Table 3) demonstrating that the C-terminal region of E1A induces pseudohyphal growth independently of the MAPK and cAMP/PKA pathways, but requires Phd1p (Figure 6). This is also in agreement with our observation that the regions of E1A that interact with Yak1p are not essential for induction of pseudohyphal growth (Figures 3 and 7).

Homologs of yeast *YAK1* have been recently cloned and characterized. These include *Drosophila MNB* (Tejedor *et al.*, 1995); *Dictyostelium YAKA* (Souza *et al.*, 1998); and mammalian *DYRK1A*, *DYRK1B*, *DYRK1C*, *DYRK2*, *DYRK3*, *DYRK4*, and *DYRK4B* (Kentrup *et al.*, 1996; Becker *et al.*, 1998). Although the precise function of the Dyrks has yet to be defined, they probably play an important role in regulating cell cycle and differentiation. *Dictyostelium YAKA* is required for the initiation of development, and overexpression of *YAKA* causes cell cycle arrest in nutrition-rich medium, promoting developmental events (Souza *et al.*, 1998). In *Drosophila*, mutation of *MNB* results in specific defects in the development of the central nervous system (Tejedor *et al.*, 1995). In humans, *DRYK1A* is located in the "Down syndrome critical region" of chromosome 21 (Chen and Antonarakis, 1997), suggesting that it too may be involved in development.

In addition to binding to Yak1p, we demonstrated that E1A interacts with rat Dyrk1A and human Dyrk1B in vitro (Figure 8), suggesting that the C-terminal domain of E1A targets a conserved sequence present in both yeast Yak1p and mammalian homologs. Importantly, the interaction of E1A with rat Dyrk1A enhanced the ability of Dyrk1A to phosphorylate itself and histone H3 in vitro (Figure 9), indicating that E1A could potentially activate Dyrk function at inappropriate times.

We determined that the interaction of E1A with Yak1p requires two separate regions spanning aa 187–221 and 241–284 of E1A (Figure 7). This region encompasses that required for the interaction of E1A with CtBP, but is more extensive. Mutants within the region spanning aa 241–284 are impaired for the ability to immortalize primary rodent cells, and fail to block ras-induced tumorigenesis and metastasis in rodent systems (Schaeper *et al.*, 1995). Unfortunately, mutants in the region spanning aa 187–221 were not tested in that study. However, the existing data suggest a possible connection between these activities in mammalian cells and the interaction of E1A with Dyrks.

In conclusion, we have identified yeast Yak1p and the mammalian Dyrk1 proteins as a new family of cellular regulatory proteins targeted by the C-terminal region of E1A. Our data suggest that Yak1p modulates Ras2p signaling to regulate yeast pseudohyphal differentiation. By analogy, the Dyrk proteins may function similarly in mammalian cells to modulate ras function. Interestingly, the targeting of mammalian Dyrks by E1A may contribute to the ability of E1A to negatively modulate ras-induced tumorigenicity and metastasis.

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