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Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*

(*Saccharomyces cerevisiae*/*cdc*/temperature-sensitive mutant/zinc/oncogene homology)

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ABSTRACT **Antibodies raised against the protein encoded by a *lacZ–CDC28* in-frame fusion were shown to immunoprecipitate the *CDC28* product from yeast cell lysates. The polypeptide p36^{CDC28} is a phosphoprotein of apparent M_r 36,000. Immune complexes prepared from yeast cell lysates by using anti-CDC28 antibody were found to possess a protein kinase activity, as determined by the transfer of label from [γ-^{32}P]ATP to a coprecipitated M_r 40,000 protein of unknown identity or function (p40). This activity was absent or thermolabile when extracts were prepared from several different *cdc28* temperature-sensitive strains. The protein kinase activity was dependent on Zn^{2+} and transferred phosphate specifically to serine and threonine residues.**

The product of the yeast gene *CDC28*, required for initiation of the cell cycle (1, 2), has been shown to share homology with a number of vertebrate protein kinases, including those of the *src* oncogene family (3). We have prepared immune sera reactive against the *CDC28* product by constructing an in-frame fusion between the *Escherichia coli lacZ* and *Saccharomyces cerevisiae CDC28* genes and using the encoded hybrid protein as an antigen (4). Employing these sera, we demonstrate that immunoprecipitates have an associated protein kinase activity, as predicted from the sequence determination, thus implicating protein phosphorylation events in the initiation of cell division in yeast.

MATERIALS AND METHODS

Yeast Strains and Media. Yeast strain 381Ga.6, congeneic with 381G (5), except that *SUP4*^{ts}, *tyr1*, and *ade2* have been eliminated and *ade6* has been inserted, was provided by Duane Jeness. Strain BF264-15D (*MAT*α, *leu2, trp1, ade1, his3*) was provided by Bruce Futcher. Mutants containing temperature-sensitive alleles *cdc28-4* and *cdc28-13* (2) were congeneic by transplacement of the mutant alleles (6) into the wild-type strain BF264-15D. Where amplification of the wild-type *CDC28* gene was required, a strain was constructed by transforming a plasmid containing the entire *CDC28* gene and surrounding sequences and a 2-μm replicon for high-copy maintenance (5) into the *cdc28* mutant strain CG(*28-4*)381Ga.6. This strain contained the allele *cdc28-4* and was congeneic with 381Ga.6. Unless specified, cells were grown in YEPD medium (1% yeast extract/2% Bactopeptone/2% glucose) supplemented with 50 mg each of adenine and uracil per liter.

Preparation of *in Vivo* Labeled Cell Lysates, Immunoprecipitations, and Electrophoretic Analysis. For labeling proteins with [^{35}S]methionine, yeast cells with or without dosage-amplifying plasmid were grown in minimal medium (7) supplemented with amino acids but lacking methionine (also lacking tryptophan for plasmid-containing cells). When a density of 10^7 cells per ml had been reached, 0.25 mCi (1 Ci = 37 GBq) of [^{35}S]methionine (New England Nuclear) was added to 5-ml cultures. After growth for 1.5 hr at 36°C, cells were harvested by centrifugation. For labeling with ^{32}P, wild-type and plasmid-containing cells were grown in Burkholder minimal medium (8) (where KCl was substituted for KH_2PO_4) supplemented with 0.1% yeast extract (Difco). When a density of 10^7 cell per ml had been reached, 0.30 mCi of [^{32}P]orthophosphate (ICN; carrier-free) was added to 5-ml cultures. After growth for 2–3 hr at 36°C, cells were harvested by centrifugation. Preparation of lysates for immunoprecipitation was the same for ^{35}S-labeled and ^{32}P-labeled cells. After harvest, cells were washed once with ice-cold water. All subsequent steps were performed between 0°C and 4°C. Pellets were suspended in 200 μl of lysis buffer, a detergent mixture containing 1% Triton X-100, 1% deoxycholate (sodium salt), 0.1% $NaDodSO_4$, and 50 mM Tris·HCl (pH 7.2), and transferred to 1.5-ml Microfuge tubes. After addition of 10 μl of Aprotinin (Sigma), cells were disrupted by mixing in a Vortex with 0.5-mm glass beads (Braun). The lysates then were clarified by centrifugation for 15 min in a Eppendorf Microfuge. Immunoprecipitation and polyacrylamide gel electrophoresis were performed as described (4). Gels containing ^{32}P-labeled samples were boiled for 30 min in 5% trichloroacetic acid to reduce the background of labeled nonphosphoprotein material. Dried gels were exposed to XAR-5 film (Kodak) at −75°C for 3 days. For ^{32}P-labeled samples, a Cronex Lightning Plus intensifying screen was used. For two-dimensional (2-D) gel analysis, *Staphylococcus aureus* protein A pellets were suspended in isoelectric focusing sample buffer and samples were subjected to 2-D gel electrophoresis essentially as described by Lörincz *et al.* (9), except that samples were loaded on the acidic ends of isoelectric focusing gels (the first dimension) rather than the basic end, the usual practice.

***In Vitro* Protein Kinase Assays.** Thirty milliliters of wild-type or congeneic mutant culture was grown to a density of 10^8 cells per ml (late logarithmic phase), harvested by centrifugation, washed once by centrifugation through 50 ml of ice-cold water, and resuspended in 1.2 ml of lysis buffer (see above). After addition of 60 μl of Aprotinin (Sigma), glass beads were added (0.5 mm; Braun) and cells were lysed by mixing in a Vortex. Three hundred microliters of lysate clarified by two 15-min spins in a Microfuge was used for each immunoprecipitation reaction. Thirty microliters of either immune or preimmune serum was added and the mixture was incubated on ice for 16 hr in a Microfuge tube. Serum was prepared by precipitation in 30% saturated ammonium sulfate, heating to 56°C for 30 min, and centrifugation to remove lipoproteins. After incubation, samples were removed to fresh tubes and 30 μl of a suspension of protein A-Sepharose

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Abbreviation: 2-D, two-dimensional.

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(75 mg/ml; Sigma) was added. Samples were mixed in a Vortex intermittently for 1 hr followed by collection of the beads by centrifugation for 10 sec in a Microfuge. Six washes were performed by suspending beads in 1 ml of RIPA buffer (10), centrifugation for 10 sec, and removal of the supernatant. An additional wash was performed in 7.5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.2. The final wash was performed in this same buffer, where no additional cation was to be added during the assay, or in the same buffer made 1 mM $ZnCl_2$ or 1 mM $CaCl_2$ where appropriate. Ten microliters of reaction mixture containing 50 μCi of [γ-^{32}P]ATP (ICN; 4000 Ci/mmol), 7.5 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.2), and 1 mM $ZnCl_2$ or 1 mM $CaCl_2$, where appropriate, was added to the pelleted beads. Reactions proceeded for 30 min at 23°C. Pellets then were washed two times with 200 μl of ice-cold buffer (7.5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.2) and either resuspended in $NaDodSO_4$/polyacrylamide gel sample buffer or 2-D gel sample buffer. One-dimensional or 2-D gel electrophoresis were performed as described above.

Phosphoamino Acid and Tryptic Fingerprint Analyses. *In vivo* ^{32}P-labeled samples were prepared from *CDC28* plasmid-containing cells, immunoprecipitated, and electrophoresed as described above except for the following changes. Two and one-half millicuries was used to label 2.5-ml cultures. The resulting cleared lysate (100 μl) was immunoprecipitated with 10 μl of serum. Resolution of immunoprecipitated proteins was by 2-D gel electrophoresis (9). *In vitro* labeled samples were prepared as described above except that 100 μl of affinity-purified antibody was incubated with 1 ml of cleared lysate. Precipitation with 30 μl of protein A-Sepharose and incubation with labeled ATP were as above. Washed immunoprecipitates were solubilized by boiling in sample buffer and electrophoresed through 7.5% polyacrylamide gels. Bands were excised from dried gels and protein was extracted as described by Kloetzer *et al.* (11). Analysis was by 2-D thin-layer electrophoresis as described by Hunter and Sefton (12).

For tryptic fingerprint analysis of phosphopeptides, samples were prepared as above. Phosphoproteins were extracted from gels, subjected to trysinolysis, and analyzed essentially as described by Hunter and Sefton (12).

RESULTS

CDC28 Immune Complexes Can Be Obtained from Yeast Cell Lysates. Antisera prepared against the product of a *lacZ–CDC28* in-frame fusion are capable of immunoprecipitating the *CDC28* product from yeast cell lysates. Wild-type cell lysates, labeled by growth in the presence of [^{35}S]methionine, yielded upon immunoprecipitation a protein of M_r ≈36,000, as determined by electrophoresis through $NaDodSO_4$/polyacrylamide gels followed by autofluorography (Fig. 1). The predicted M_r of the *CDC28* gene product based on DNA sequence analysis is 34,000 (3), a reasonable correspondence. The identity of the M_r 36,000 protein as the *CDC28* product, however, was confirmed by analyzing cell lysates prepared from *CDC28* plasmid-containing cells where the dosage of the gene was expected to be amplified considerably (5). The anticipated 20-fold increase in immunoprecipitated product is seen in Fig. 1, lane 3. Thus, it is likely that the M_r 36,000 band in Fig. 1 corresponds to the product of the *CDC28* gene (p36^{CDC28}). Another protein of apparent M_r 70,000 (p70) was specifically immunoprecipitated (Fig. 1, lanes 1 and 3). Because it showed no response to *CDC28* gene dosage, we assume it to be a cross-reactive species encoded by a different gene. It was observed in the course of this experiment that *CDC28* product was solubilized and immunoprecipitable only when cells were lysed in buffers containing low concentrations of salt.

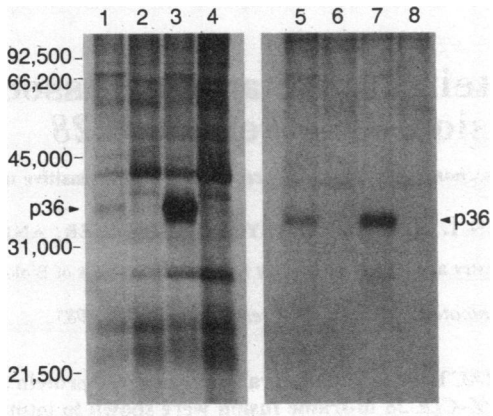

FIG. 1. Antibodies prepared against a *CDC28–lacZ* fusion protein immunoprecipitate the *in vivo CDC28* product. Immunoprecipitates were prepared, subjected to electrophoretic analysis, and autoradiographed. Lanes 1–4, ^{35}S-labeled samples; lanes 5–8, ^{32}P-labeled samples: lanes 1 and 5, immune serum, wild-type cells; lanes 2 and 6, preimmune serum, wild-type cells; lanes 3 and 7, immune serum, *CDC28* plasmid-containing cells; lanes 4 and 8, preimmune serum, plasmid-containing cells. The position of the *CDC28* product (p36^{CDC28}) is indicated. Standards used as molecular weight markers: muscle phosphorylase b, M_r 92,500; serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500.

The *CDC28* Product Is a Phosphoprotein. Based on the homology of the *CDC28* product to protein kinases, most of which are isolated as phosphoproteins, it was of interest to determine whether p36^{CDC28} likewise was a phosphoprotein *in vivo*. Lysates of cell cultures labeled by growth in medium containing [^{32}P]orthophosphate were subjected to immunoprecipitation. Immune complexes then were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. The results shown in Fig. 1, lanes 5–8, indicate that p36^{CDC28} is a phosphoprotein.

To determine the target amino acids for *in vivo* phosphorylation of the *CDC28* product, bands analogous to those shown in Fig. 1 were excised from gels and protein was extracted. Partial acid hydrolysis and thin-layer electrophoresis indicate that p36^{CDC28} contains both phosphothreonine and phosphoserine (see Fig. 4*F*). Overexposure of the electropherogram yielded a faint spot at the position of phosphotyrosine (data not shown). It is not clear whether this corresponds to a legitimate minority species or to an artifact of the analysis.

CDC28 Immune Complexes Contain a Zn^{2+}-Dependent Protein Kinase Activity. When immune complexes, prepared as described above, were incubated with [γ-^{32}P]ATP, incorporation of phosphate into a M_r 40,000 protein was observed (Fig. 2*A*, lanes 1 and 2). Reactions run with preimmune complexes showed no such incorporation. The origin of p40 is not known. It is, however, assumed to be contained in p36^{CDC28} immune complexes and, as such, may correspond to a high-affinity *in vivo* substrate of this putative protein kinase. Labeling of another protein of apparent M_r 20,000 was observed to a variable extent. Comparison of phosphopeptides of the two proteins by tryptic fingerprinting suggested that this M_r 20,000 protein is related to p40 by proteolysis (data not shown). Since neither p40 nor p20 is evident in *in vivo* labeled immunoprecipitates (Fig. 1), they must be present in such at levels substoichiometric to p36^{CDC28}. We cannot exclude the possibility that p40 is a tightly bound but fortuitous contaminant.

The assays shown in Fig. 2*A*, lanes 1 and 2, were performed in the presence of 1 mM Zn^{2+}. Without this cation, incorporation of phosphate into a large number of proteins was observed for both immune and preimmune complexes (Fig.

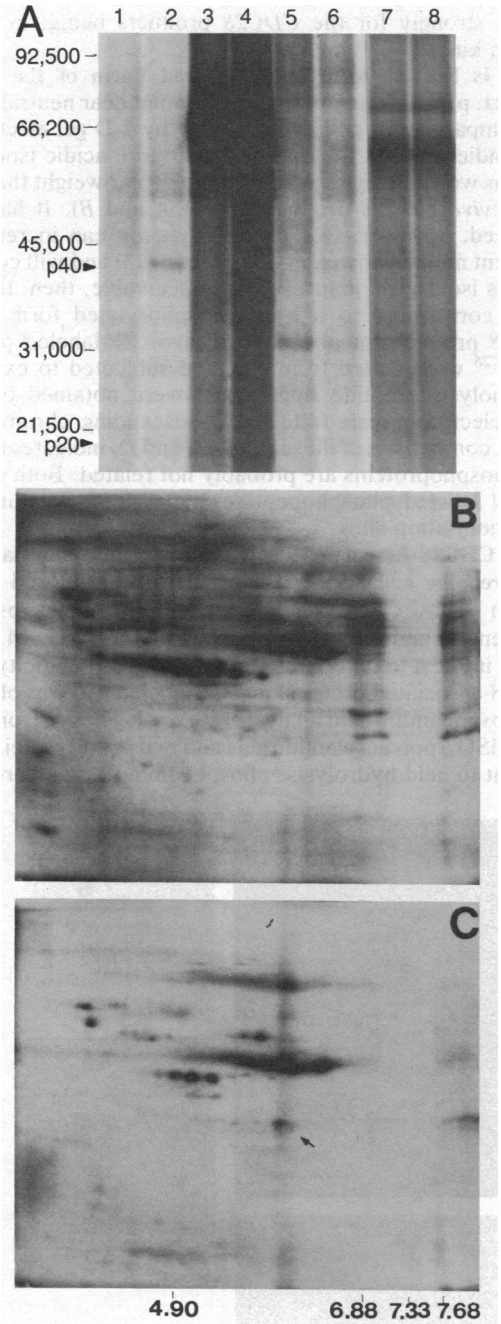

FIG. 2. *In vitro* protein kinase activity of *CDC28* product immune complexes. Immune complexes prepared by using anti-p36^{CDC28} serum and lysates from wild-type yeast strain BF264-15D were incubated with [γ-^{32}P]ATP in order to assay for protein kinase activity. (*A*) Phosphorylated proteins from these reactions were compared by electrophoresis through NaDodSO$_4$/7.5% polyacrylamide gels. Dried gels were exposed to x-ray film (Kodak XAR-5) in the presence of an intensifying screen for 2 days. Lanes 1 and 2, 1 mM $ZnCl_2$ + 7.5 mM $MgCl_2$, preimmune and immune serum, respectively; lanes 3 and 4, 7.5 mM $MgCl_2$, preimmune and immune serum, respectively; lanes 5 and 6, 1 mM $CaCl_2$ + 7.5 mM $MgCl_2$, preimmune and immune serum, respectively; lanes 7 and 8, no additional cation initially, addition of 1 mM $ZnCl_2$ after 15 min to sample shown in lane 7 and additional incubation of 15 min. Molecular weight standards are as in Fig. 1. The positions of bands corresponding to p40 and p20 are indicated. (*B*) Phosphorylated proteins from anti-p36^{CDC28} immunoprecipitate reaction containing no additional cation separated by 2-D gel electrophoresis. (All reactions contained 7.5 mM $MgCl_2$.) Electrophoresis was performed essentially as described by Lörincz *et al.* (9), except that samples were loaded on the acidic ends of isoelectric focusing gels (the first dimension) rather than the basic end. Dried 2-D gels (10%) were exposed with an intensifying screen for 1 week. (*C*) Phosphorylated proteins from anti-*CDC28* product immunoprecipitate reaction containing 1 mM $ZnCl_2$ separated by 2-D gel electrophoresis. Electrophoresis was as described for *B* above. The arrow indicates the position of p40. Isoelectric focusing standards were bovine serum albumin, pI = 4.90; horse myoglobin, pI = 6.88 and 7.33; sperm whale myoglobin (minor species), pI = 7.68.

2*A*, lanes 3 and 4). Under these conditions, it was difficult to resolve specific incorporation into p40 by one-dimensional electrophoresis. Protein kinase assays performed with and without Zn^{2+}, therefore, were compared by 2-D gel electrophoresis (Fig. 2 *C* and *B*, respectively). Although Zn^{2+} strongly inhibits incorporation of phosphate into a large number of proteins, it apparently is also required for incorporation into p40. Precise superposition of the electropherograms shown in Fig. 2 *B* and *C* indicates that none of the faint spots seen in Fig. 2*B* comigrates with p40. One millimolar Ca^{2+} was found to have a general inhibitory effect on incorporation of phosphate into proteins but was not efficient in stimulating incorporation into p40 (Fig. 2*A*, lanes 5 and 6). It appears, therefore, that insolubilized immune complexes prepared from highly concentrated yeast lysates are contaminated nonspecifically with protein kinase activity that is strongly inhibited by both Zn^{2+} and Ca^{2+}. Incorporation into p40 is, on the other hand, strongly stimulated by Zn^{2+}.

The possibility that the predominant effect of Zn^{2+} is the stimulation of a protein phosphatase to which phosphorylated p40 is resistant was excluded by incubating immune complexes first without Zn^{2+} and then adding Zn^{2+} for a subsequent period of incubation. It was observed that no loss of phosphate labeling of proteins occurred upon the addition of Zn^{2+} (Fig. 2*A*, lanes 7 and 8).

Because Zn^{2+} was required to demonstrate specific protein kinase activity, $ZnCl_2$ was included at a concentration of 1 mM in all subsequent assays. The rationale for screening Zn^{2+} as a potential effector in this assay will be discussed below.

Effects of Temperature-Sensitive *cdc28* Mutations on Protein Kinase Activity. A large number of temperature-sensitive alleles of *CDC28* have been accumulated and characterized (2). It was anticipated that some of these would encode labile proteins that would be useful probes of the specificity of our assay. When immune complexes from wild-type and an extremely temperature-sensitive mutant strain carrying the allele *cdc28-4* were compared, strong specificity was observed (Fig. 3, lanes 1 and 2). Incorporation of phosphate into p40 was eliminated when immunoprecipitates were prepared from the *cdc28-4* lysate and assayed at the permissive temperature. When wild-type and *cdc28-4* mutant cells were mixed prior to preparation of the lysate, specific kinase activity was retained (Fig. 3, lane 3), arguing against the likelihood that loss of activity in the mutant lysate is caused by the action of a *trans*-acting inhibitor of protein kinase function or the action of a specific phosphatase. The inability to detect protein kinase activity in lysates prepared from *cdc28-4* mutant cells even at the permissive temperature must be a result of impaired function rather than of increased turnover of the mutant polypeptide, as the latter can be immunoprecipitated at wild-type levels (data not shown).

When mutant extracts were prepared by using another mutant allele, *cdc28-13*, protein kinase activity was observed at wild-type levels using the standard assay (Fig. 3, lanes 4 and 5). However, when immune complexes were preincubated at 38°C for 40 min prior to assay, which was also performed at 38°C, protein kinase activity for the mutant immune complexes was labile compared to wild type (Fig. 3, lanes 7 and 8). In the case of another mutant allele, *cdc28-9*, activity at 38°C was reduced compared to wild type but not eliminated entirely as for *cdc28-13* (data not shown). *In vitro*

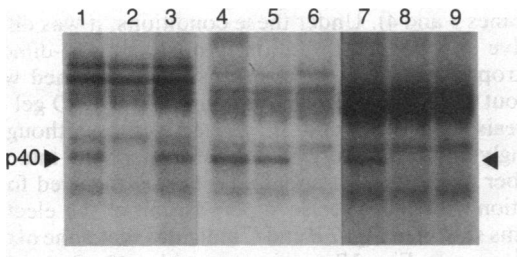

FIG. 3. Thermolabile protein kinase activity from temperature-sensitive *cdc28* mutants. Lysates were prepared from wild-type strain BF264-15D and two congeneic *cdc28* mutant strains. *CDC28-4* and *cdc28-13* correspond to the mutant alleles (4). Cultures were grown at 23°C and lysates were prepared and immunoprecipitated with anti-*CDC28* product immune serum as described in the legend to Fig. 2. Lanes 1, 2, and 3, wild type, *cdc28-4*, and a mixture of the two, respectively, all assayed at 23°C. Lanes 4, 5, and 6, wild type, *(cdc)28-13*, and *cdc28-4*, respectively, again assayed at 23°C. Lanes 7–9, duplicate immunoprecipitates (equivalent to lanes 4–6) preincubated at 38°C and assayed at 38°C. Film exposure has been normalized so that wild-type lanes (4 and 7) show bands of approximately equivalent intensity. In fact, wild-type band intensity was reduced by ≈50% after high-temperature incubations.

phosphorylation of p40, therefore, shows *in vitro* thermolability for two temperature-sensitive alleles as well as absolute lability for a third. In light of the assignment of the *CDC28* product to the protein kinase family of proteins based on predicted primary structure homology (3), this lability of protein kinase activity *in vitro* for mutant immune complexes argues strongly for the *CDC28* products being, in fact, a protein kinase.

p40 Is Not an Autophosphorylated Form of the *CDC28* Product. $p36^{CDC28}$ has an isoelectric point near neutrality (pH 7). Comparison of p40 and $p36^{CDC28}$ by 2-D gel electrophoresis indicates that the former has a more acidic isoelectric point as well as a higher apparent molecular weight than does the *in vivo CDC28* product (Fig. 4 *A* and *B*). It has been observed, however, that phosphorylation can increase the apparent molecular weight of a protein (13) and will certainly alter its isoelectric point. It was conceivable, then, that p40 might correspond to a hyperphosphorylated form of the *CDC28* product not observed *in vivo*. ^{32}P-labeled p40 and $p36^{CDC28}$ were eluted from gels and subjected to extensive trypsinolysis. Peptide fingerprints were obtained by thin-layer electrophoresis followed by ascending chromatography. A comparison of these (Fig. 4 *C* and *D*) indicates that the two phosphoproteins are probably not related. Both contain several labeled phosphopeptides, consistent with multiple phosphorylation sites.

The CDC28-Associated Protein Kinase Phosphorylates p40 on Threonine and Serine. Because of the proposed role of protein kinases that have a specificity for tyrosine in oncogenesis and the action of growth factors (12, 14, 15), it was of interest to determine the amino acid specificity of the CDC28-associated protein kinase. Labeled p40 was obtained for phosphoamino acid analysis by excising bands from dried $NaDodSO_4$/polyacrylamide gels and extracting protein. Subsequent to acid hydrolysis, phosphoamino acids were sepa-

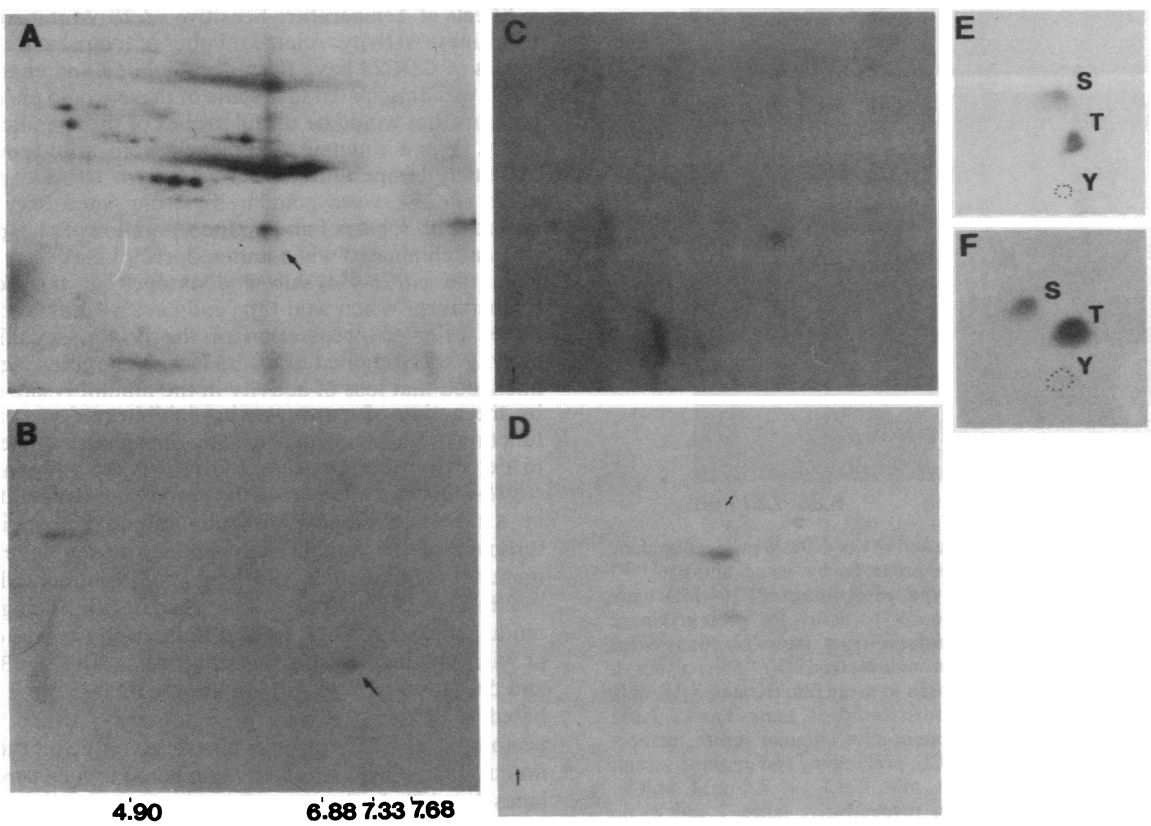

FIG. 4. Comparison of p40 labeled *in vitro* and $p36^{CDC28}$ labeled *in vivo*. (*A*) An *in vitro* phosphorylation assay was performed and subjected to 2-D gel analysis (as in Fig. 2*B*). Arrow indicates position of p40. (*B*) $p36^{CDC28}$ was ^{32}P-labeled and immunoprecipitated as described in the legend to Fig. 1, lane 5, and 2-D gel analysis was performed. pI markers are the same as in Fig. 2. Arrow indicates position of $p36^{CDC28}$. (*C*) p40 subjected to tryptic fingerprint analysis. Arrow indicates position of sample application. Electrophoresis (the first dimension) was to the right and ascending chromatography (the second dimension) was in the direction of the arrow. (*D*) $p36^{CDC28}$ labeled with ^{32}P *in vivo* subjected to tryptic fingerprint analysis. Application of sample was at position of the arrow. Electrophoresis and chromatography were as in *C*. (*E*) Phosphoamino acid analysis of p40 labeled *in vitro*. Positions of phosphoamino acids were identified by using unlabeled standards and staining plates with ninhydrin. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Exposure with an intensifying screen was for 2 weeks. (*F*) Phosphoamino acid analysis of $p36^{CDC28}$ labeled *in vivo*.

rated by 2-D thin-layer electrophoresis and visualized by autoradiography (Fig. 4*E*). Both phosphothreonine and phosphoserine are observed, indicating a threonine/serine specificity rather than a tyrosine specificity. Recently, several vertebrate oncogenes whose products were shown to be related to protein kinases based on DNA sequence analysis were shown actually to encode protein kinases of the threonine/serine type. These include v-*mos* (11), v-*raf*, and v-*mil* (16).

Phosphoamino acid analysis of $p36^{CDC28}$ labeled *in vivo* shows this protein, as well, to be a target of a threonine/serine-type protein kinase (Fig. 4*F*). Although there is no evidence of autophosphorylation *in vitro*, such a mechanism for phosphorylation *in vivo* cannot be excluded.

DISCUSSION

We have demonstrated a protein kinase activity in immune complexes prepared with anti-*CDC28* product antibody. The activity is not present when preimmune serum is used. Furthermore, this activity can be shown to be absolutely labile or thermolabile for three different temperature-sensitive mutant alleles. The simplest interpretation of these data is that the *CDC28* gene product encodes the protein kinase responsible for the observed phosphorylation events. However, alternative interpretations are consistent with our results. We feel that the other immunoprecipitated species (p70) is excluded as the source of p40 phosphorylation in face of the results with *cdc28* temperature-sensitive mutants. It is possible, though, that the *CDC28* product coprecipitates as a complex containing p40 as well as an active protein kinase and that mutant lability in the assay results from dissociation of the complex rather than from lability of protein kinase activity. The most compelling argument against this model is the homology of the *CDC28* product, at the level of primary structure, to known protein kinases. In effect, a protein kinase activity is predicted. Furthermore, the stimulatory effect of zinc is consistent with putative zinc binding sites predicted for the *CDC28* product based on primary and secondary structure analysis (see below).

To our knowledge, a protein kinase activity that is stimulated by cationic zinc has not been reported previously. It is not clear, at this time, what role zinc plays in the function of this enzyme *in vivo*. It is conceivable that other cations are utilized in the intracellular milieu. Calcium appeared to function weakly in this capacity *in vitro*, although it is not yet known whether the weak band seen in Fig. 2, lane 6, is p40. On the other hand, the assay conditions were arbitrary (1 mM Ca^{2+}). It is possible that this concentration is far from optimal. The rationale for initially screening for a zinc effect was the discovery of two probable zinc chelating sites in the course of structural analysis of the predicted *CDC28* product sequence. These correspond to residues 78–82 (His-Ser-Asp-Ala-His) and to residues 130–134 (His-Arg-Ile-Leu-His), which a secondary-structure prediction analysis by the method of Chou and Fasman (17) suggests are in α-helical configurations (data not shown). Should the imidazoles of either or both of these domains be exposed to solvent, they would constitute sites of zinc chelation (18). The observation that the *CDC28* product-associated protein kinase activity is stimulated by zinc cation suggests that such a physical interaction does probably occur.

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