Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 301-309

FISSION YEAST CDC25 IS A CELL-CYCLE REGULATED PROTEIN

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Received January 25, 1990

Fission yeast cell division is initiated by the cdc2/cdc13-cyclin protein kinase which in its catalytically active state comprises the mitotic inducer. During interphase the cdc2/cyclin complex is assembled in an inactive state that requires *cdc25*$^+$ gene function for M-phase activation. The *cdc25*$^+$ product, a 76 kd phosphoprotein, is shown to oscillate in abundance during the cell cycle, reaching a peak at G2/M, and to be sensitive to nitrogen starvation. The level of cdc25 is subject to feedback regulation involving both cdc25 and cdc2. © 1990 Academic Press, Inc.

Genetic investigation of the initiation of mitosis in fission yeast has focused on *cdc2*$^+$ and other genes such as *suc1*$^+$ (1,2), *cdc13*$^+$ (3,4), *cdc25*$^+$ (5), *wee1*$^+$ (6), *bws1*$^+$ (7) and the six *mcs*$^+$ genes (8) with which *cdc2*$^+$ closely interacts. *cdc2*$^+$ encodes a 34 kilodalton (kd) protein kinase that is required both for the initiation of DNA synthesis and also for entry into mitosis (9-11). *cdc13*$^+$ encodes a mitotic cyclin that, in common with cyclins in multicellular organisms (12,13), is degraded at each cell division (14-17). Molecular investigation of the *cdc13*$^+$-encoded cyclin suggests that it acts as an essential M-phase subunit of the cdc2 protein kinase that may determine both the substrate specificity and nuclear localization of the enzyme (16). A further essential subunit of the protein kinase is the 13 kd product of *suc1*$^+$ (18), a gene that was isolated as a plasmid-borne sequence that could rescue some ts alleles of *cdc2* (1).

During interphase, the fission yeast cyclin is synthesized continuously and, with the *suc1*$^+$ protein, forms a complex with cdc2 (16). However, this multimolecular complex is not active as an M-phase inducer until execution of the *cdc25*$^+$ gene function (16). *cdc25*$^+$ is essential for the initiation of mitosis (3), except in certain mutants that cause premature initiation of mitosis (5). These include dominant alleles of *cdc2* itself (cdc2-w mutants, ref. 19) and loss-of-function mutations of the *wee1* gene (5). It has been proposed that *cdc25*$^+$ and *wee1*$^+$ act antagonistically, respectively as an activator and inhibitor of the cdc2 protein kinase (5,20,21). Recently, two homologous genes have been identified in *Drosophila* (22) and in budding yeast (23), emphasizing the generality of this regulatory pathway. Here we have studied the properties of the *cdc25*$^+$ gene product and showed that it is a cell cycle regulated protein.

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0006-291X/90 $1.50
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Results

cdc25 gene product

In order to study the properties of the *cdc25⁺* protein the gene was first isolated by marker rescue of a *cdc25-22* mutant. The restriction map of the sequence obtained was in accord with that described previously (20). Full length *cdc25⁺* product was expressed in *E. coli* and a rabbit was immunized with purified protein. Affinity purification of the serum (referred as B1-Af) was achieved by passing it through a column to which bacterially-synthesized cdc25 was covalently coupled (see Experimental Procedures).

To determine whether these sera specifically recognized fission yeast *cdc25⁺* an overexpressing strain and a disruptant strain were constructed (see Experimental Procedures). Overexpression of *cdc25⁺* has previously been described as conferring a wee phenotype (20). However, in multiple independent yeast isolates the phenotype we observed was one of heterogeneity in cell length at division, when cells were cultured in yeast extract containing medium (Fig. 1B, top panel), rather than a distinct wee phenotype. In minimal medium a more distinct wee phenotype was apparent (Fig. 1B, bottom panel).

Extracts were prepared from wild-type, overexpressing (*cdc25-OP*) and disruptant strains and probed with affinity-purified anti-cdc25 sera in immunoblots. Efficient

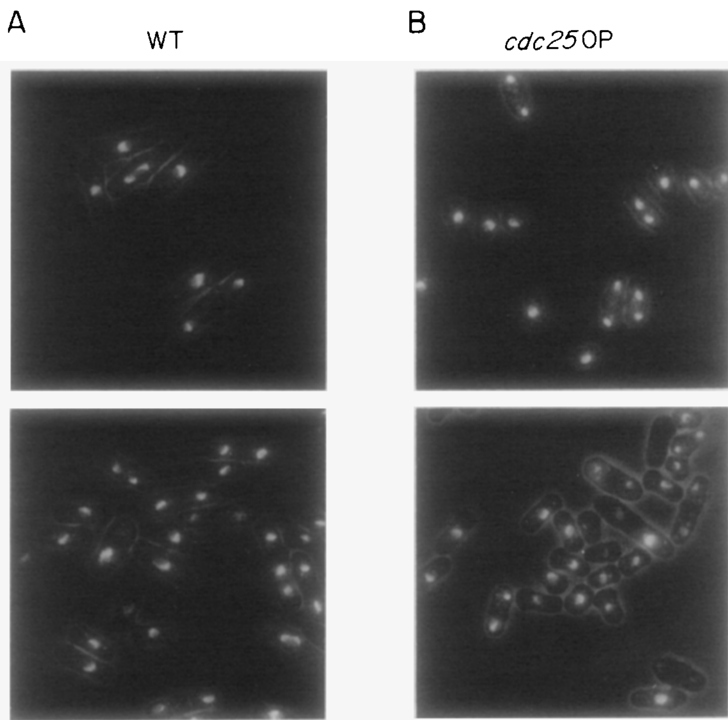


Fig. 1. Overexpression of *cdc25⁺* gene product.
Morphology of DAPI-stained wild-type (A, WT: 972) and cdc25-overexpressing (B, *cdc25-OP*: SP868) strains, cultured at 25°C in minimal medium (top panels) or yeast extract-containing medium (lower panels).

release of immunologically reactive material required harsh extraction conditions (8M urea, see Experimental Procedures). Low levels of a cross-reactive species of 76 kd apparent molecular weight (slightly greater than the 67 kd expected size of cdc25) was observed in extracts of the wild-type strain. Much higher levels of this protein were apparent in *cdc25-OP* (Fig. 2A) and no signal was detectable in the gene disrupted strain. Cell lysates from exponentially growing culture of overexpressing, disruptant and wild-type strains were subjected to immunoprecipitation (B1-Af) after *in vivo* ^{32}P labelling (Fig 2B). A phosphoprotein was immunoprecipitated at a similar molecular weight as the one observed by western blot. Furthermore, this protein was more abondant in the overexpressing strain and absent in the disruptant. We conclude that this polypeptide is the yeast cdc25 protein. Making use of bacterially-synthesized cdc25 at known protein concentrations we have estimated that in actively proliferating wild-type yeast, cdc25 comprises 0.005% of total cellular protein. In the overexpressing strain the abundance is approximately 20-fold greater.

cdc25 cell-cycle oscillation

At present, the only fission yeast cell-cycle regulatory protein that has been shown to vary in abundance during the division cycle is the *cdc13⁺*-encoded cyclin (16). Since *cdc25⁺* is also involved in the initiation of mitosis we investigated the level of its product at the onset of mitosis. A *cdc25-22* temperature-sensitive strain was used to obtain a culture synchronized in G2 (24). After 4.25 hours at the restrictive temperature (36°C), division arrest was reversed by rapid transfer to a permissive temperature (25°C). The cell plate index reached 80% in the first cycle after release

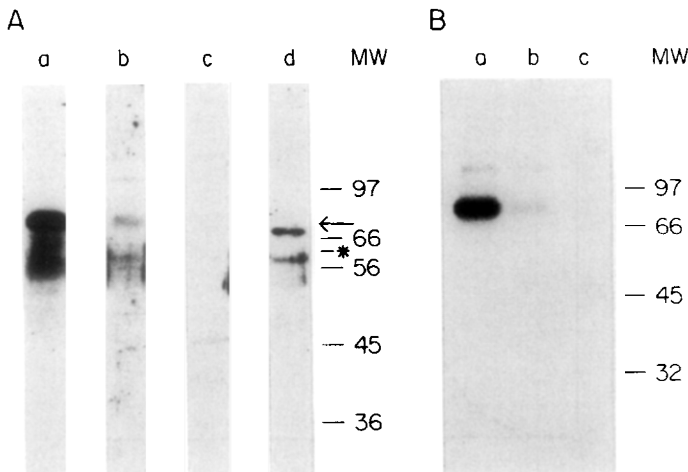
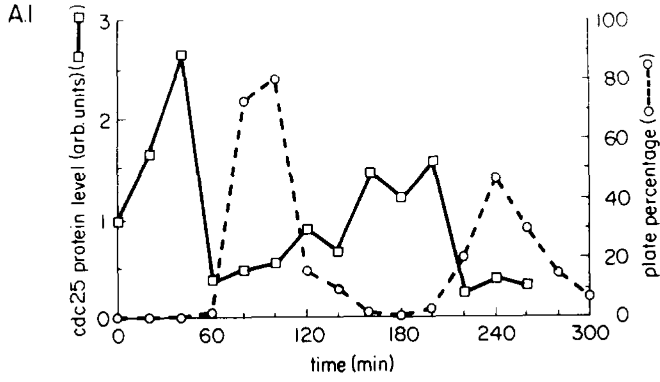
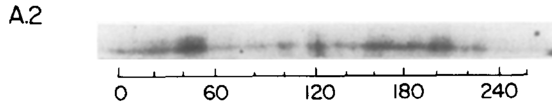


Fig. 2. Identification of *cdc25⁺* gene product.
(A) Immunoblots (200 µg total protein) of extracts from overexpressing (lane a), wild type (lane b) and disruptant (lane c) strains probed with anti-cdc25 antibodies (B1-Af). Lane c, is 50 ng of bacterial purified cdc25. The arrow indicates the cdc25 protein and the star indicates the major degradation product.
(B) Immunoprecipitation of cdc25 after ^{32}P *in vivo* labelling from overexpressing (lane a), wild type (lane b) and disruptant (lane c) strains.

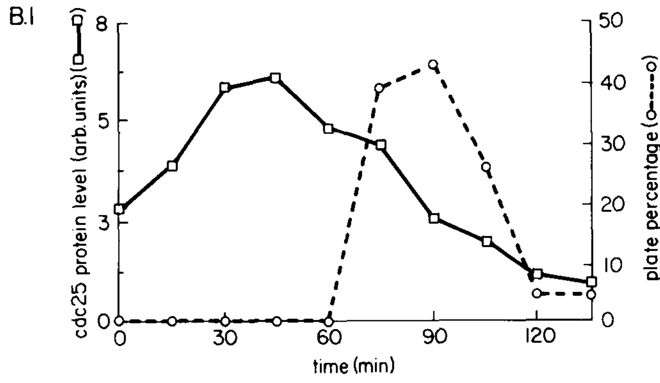
A.1



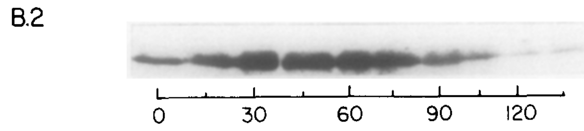
A.2



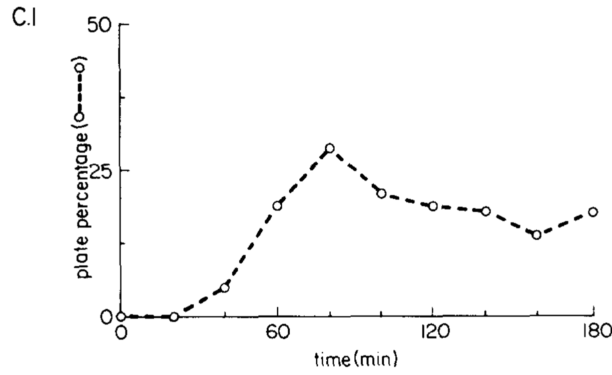
B.1



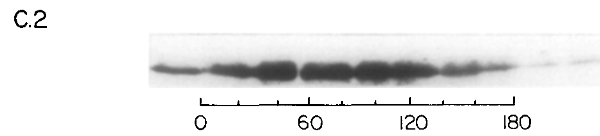
B.2



C.1



C.2



(Fig. 3A. 1). Samples were taken every 20 minutes during the 5 hours after release, cell extracts were prepared and the level of cdc25 was assessed by immunobloting (Fig. 3A. 2). The relative abundance of cdc25 was determined by quantitation of the immunoblot signal (Fig. 3A. 1, see Experimental Procedures). At the point of arrest, the level of cdc25 was low but detectable. Following release, cdc25 increased to a maximum after 40 minutes and then declined very sharply 30 minutes before the peak of cell plate index (note that cell plate formation is a post-mitotic event, in the sense that nuclear division has been completed). The cell-cycle oscillation in cdc25 level was also accompanied by an alteration in mobility of the protein in SDS-PAGE (Fig. 3A. 2 and Fig. 3C. 2). The lower mobility species, most apparent forty minutes after block-release, might be a phosphorylated form of the protein since *in vivo* cdc25 is a phosphoprotein.

In the preceding experiment a *cdc25* mutant was used to study the cell cycle oscillation of the *cdc25*$^+$ product. The increased abundance of the protein shortly after release of the mutant from a restrictive temperature suggests that the level of cdc25 is regulated by a feedback loop of which cdc25 itself is a positive component (see Discussion). To exclude the possibility that the cdc25 oscillation might be an artifact of the use of the *cdc25-22* mutant the experiment was repeated using a *cdc2-33* strain. A *cdc2-33 cdc25-OP* strain was constructed in order to test whether the cell cycle oscillation of cdc25 was also sustained in the overproducer. The culture was arrested at 36°C and after 4.25 hours released at 25°C (note that *cdc25-OP* does not rescue *cdc2-33*). The level of cdc25 was followed by immunoblotting and quantified. The degree of cell cycle synchrony of this strain following release was not as good as that of the *cdc25* mutant (Fig. 3B. 1), but the abundance of cdc25 displayed cell cycle oscillation (Fig. 3B. 1 and 3B. 2). This experiment demonstrates that even at elevated levels cdc25 is subject to cell cycle regulation.

To confirm this observation, the level of cdc25 was examined in a culture synchronized by reinoculation of the smallest cells (mostly G1) in fresh complete medium, after centrifugation elutriation. Because of the low level of cdc25 protein detectable in a wild type background, an overexpressing strain (cdc25-OP) was used. The level of cdc25 protein monitored by immunoblotting was very low in small cells after elutriation and progressively increased with increasing cell size (Fig. 3C). Since in this strain the cells are heterogenous in size at mitosis (see figure 1), the obtained synchrony was poor and no obvious decrease of the protein level was observed. But this

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Fig. 3. Cell cycle oscillation of cdc25
(A) *cdc25-22* (SP32) was arrested at 36°C for 4.25 hours, then shifted down to 25°C. Every 20 minutes after release an aliquot of cells were taken for quantitative anti-cdc25 immunoblotting and determination of cell plate index (A. 1). The immunoblot (200 µg total protein) is shown in A.2.
(B) *cdc2-33 cdc25-OP* double-mutant was arrested at 36°C for 4.25 hours and released at 25°C. Samples were taken for anti-cdc25 immunoblotting (200 µg total protein) (B.2) at 15 minutes intervals. In this experiment only the first cycle after release was followed.
(C) Variation of cdc25 after centrifugation elutriation in *cdc25-Op* strain. Samples were taken every 20 min for immunoblotting (C. 2) with antiserum B1-Af (150 µg total protein) and for determination of cell plate index (C.1).

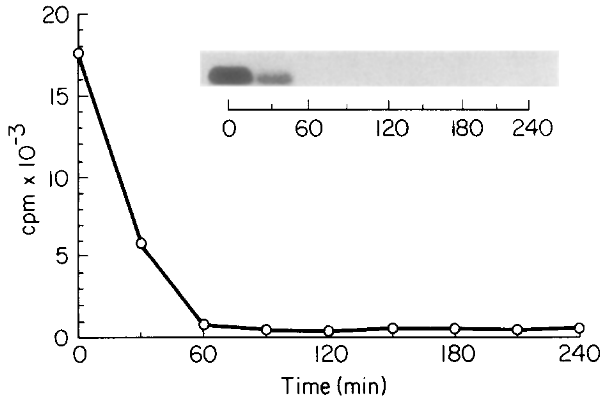

Fig. 4. Instability of cdc25 during nitrogen starvation
A culture of *cdc25-OP* (SP868) was transferred to minimal medium lacking nitrogen. Samples were taken every 30 minutes for quantitative anti-cdc25 immunoblotting (200 μg total protein) (inset shows the immunoblot with B1 antiserum).

observation confirm that cdc25 protein level is low in G1 and increases along the cell cycle.

We further tested the properties of cdc25 during entry into stationary phase under conditions of nitrogen starvation. A *cdc25-OP* strain was transferred to nitrogen-free minimal medium and the level of cdc25 was assessed by immunoblotting at intervals thereafter. In the absence of a nitrogen source the abundance of cdc25 dropped abruptly and within an hour was barely detectable (Fig. 4).

Discussion

Both cdc25 and the *cdc13*+-encoded mitotic cyclin oscillate during the fission yeast cell cycle (Fig 2 and ref. 16). The timing of degradation of the cyclin has been established with some precision and corresponds to the onset of anaphase (16,17). cdc25 is also an unstable protein that appears to be degraded in a cell-cycle dependent manner, but the relative timing of cyclin and cdc25 degradation remains to be determined with accuracy. Although cdc25 is clearly an unstable protein, its abundance does not appear to be controlled entirely by its abrupt degradation in mitosis. In both *cdc25* and *cdc2* mutants arrested at a restrictive temperature the abundance of cdc25 is low. By contrast, cyclin accumulates to high levels, even in cells arrested in G1 (16). The cdc25 protein accumulates rapidly after release of *cdc2* or *cdc25* mutants from a restrictive temperature and this process is unlikely to be regulated transcriptionally since it occurs in a strain in which the *cdc25*+ gene is driven by the constitutive *adh* promoter (Fig. 2). In addition, no systematic variation of cdc25 mRNA (data not shown) has been observed during the cell cycle. These observations suggest that the level of cdc25 protein is regulated not only by abrupt mitotic degradation, but also possibly by control of either its synthesis or stability during interphase. In such a pathway, cdc2 and cdc25 are activating components. Active cdc2 might, for example, stimulate the translational

efficiency of *cdc25⁺* mRNA, thus increasing the abundance of cdc25, leading to further activation of cdc2. The rapid decrease of cdc25 under nitrogen starvation also indicates that nutritional control affects cdc25 level regulation. In the early *Drosophila* embryo the transcript of the *string* gene, that shares homology with *cdc25⁺*, is present as a maternal message until the mid-blastula transition (22). Following this transition, the transcript is abruptly lost and subsequent transcriptional regulation of the gene appears to determine the spatially patterned timing of later embryonic cell divisions (22). These observations suggest that in the developmental context of Drosophila embryogenesis the abundance of the *string* product is likely to be regulated at least in part at the transcriptional level. The present experiments show that the abundance of the cdc25 protein oscillates in the cell cycle of fission yeast, even in a cell in which the *cdc25⁺* gene is driven by a constitutive promoter. In different organisms the *cdc25⁺* gene and its product are likely to be subject to regulation at multiple levels.

Prior to the execution point of cdc25, a cdc2/cyclin complex has formed but is not fully active as a protein kinase (16,17). Following release of a *cdc25* mutant, the abundance of cdc25 increases rapidly and cdc2 becomes tyrosine dephosphorylated and activated as a histone H1 kinase (this report,16,25, our unpublished data). cdc25, which does not share sequence homology with known phosphatases (20) might act as a "docking factor" that specifically targets a phosphatase to the cdc2/cyclin complex. The *wee1⁺* gene may be relevant to the regulation of cdc2 by phosphorylation. Although *cdc25⁺* is an essential gene in a wild-type yeast, it is not required in either *cdc2-w* or loss-of-function *wee1* mutants, each of which cause cell division to initiate prematurely (19). *wee1⁺* encodes a predicted polypeptide that shares homology with serine/threonine protein kinases (21). If *wee1⁺* indeed encodes a protein kinase, loss of which renders *cdc25⁺* unessential for the initiation of mitosis, it is likely that the *wee1⁺* protein functions, either directly or indirectly, to phosphorylate the sites in cdc2 that cdc25 later causes to be dephosphorylated.

Materials and methods

Yeast strains, cultures and cell cycle synchronization

The *S. pombe* strains used in this study were the wild type 972 (h^{-s}) and the temperature sensitive mutants *cdc25-22* (SP32) and *cdc2-33* (SP352) (3,5,19). The strain overexpressing *cdc25⁺* (SP868) was created as follows: An NdeI site was inserted at the initiating methionine of *cdc25⁺*. The NdeI-XbaI fragment was then inserted dowstream the *S.pombe adh* promoter, cloned between the SphI and the NdeI sites in the pART5 vector. The resulting plasmid, named pcdc25-5, was integrated in the yeast chomosome. SP867 is a strain in which the *cdc25* gene has been disrupted by insertion of the ura4⁺ gene in the ORF between the ClaI and the BglII sites, but carrying a *wee1-50* ts mutation to allow gowth at restrictive temperature in the absence of a functional *cdc25⁺* gene. Cells were cultured in complete medium (YEA: 0.5% yeast extract, 3% glucose, 75 µg/ml adenine) or in minimal medium (PMA)(26). For the nitrogen starvation experiments exponentially growing cells were washed twice and transferred to minimal medium without ammonium chloride. For the *in vivo* phosphorylation experiments cells were washed twice and grown 3 hours in the presence of ^{32}P (2 mci for 10^7 cells) in YE from which inorganic phosphate had been precipitated (27). Synchronous cell cultures of *S. pombe* were obtained by centrifugation elutriation

exactly as described in (18) or by arresting an exponentially growing culture of *cdc2-33* or *cdc25-22* ts mutants for 4.25 hours at the restrictive temperature of 36°C (24).

cdc25 protein purification from E. coli and antibody production

The *cdc25+* gene was cloned into the expression vector pRK171a and expressed in the *E. coli* strain BL21(DE3) (28). The cells were cultured in LB medium in the presence of ampicillin and the production of cdc25 was induced by addition of 0.4mM IPTG to the exponentially growing culture. cdc25 protein was purified as follows: the insoluble fraction of a lysozyme treated bacterial pellet was extracted twice with 0.5 % deoxycholate. Urea was then added at the final concentration of 8M and the sample was incubated for 30 min at 37°C. Further purification of the solubilized proteins was achieved by passage through a DE52 column which did not retain cdc25. After concentration of the column flow-through on Centriprep (Amicon), the sample was subjected to preparative SDS-polyacrylamide electrophoresis and proteins were extracted by incubation of the excised gel pieces in PBS (phosphate saline buffer) - 0.1% SDS at 37°C for 12 hours. After centrifugation, the protein was concentrated and used for immunization. A New Zealand white rabbit (B1) was immunized by intralymphonode injection of 50µg purified protein in complete Freund's adjuvant every two weeks and bleeds were taken ten days after each injection. Affinity purified serum (B1-Af) were obtained by chromatography on purified cdc25 coupled to CNBr-activated Sepharose 4B (Pharmacia), prepared following the instructions of the manufacturer.

Electrophoresis and immunoblots

Cells were broken with glass beads in 100 µl of buffer I (50 mM Tris-HCl pH 8.0, 1mM EGTA, 5mM EDTA, 8M urea and the following inhibitors of proteases: 0.1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor , 1 µg/ml aprotitin and 10µg/ml TPCK (tosyl phenyl-alanine chloromethyl ketone). The soluble protein fraction was recovered by centrifugation. After determination of protein concentration according to (29) samples were boiled 3 min in Laemmli sample buffer (30) and proteins were separated electrophoretically using a 7.5%-15% gradient SDS-polyacrylamide gel (30). The gels were transferred to nitrocellulose (Schleicher and Schuel, 0.1 µm) in a transfer apparatus (ABN). Filters were blocked with PBS containing 3% non-fat dry milk for 2 hours at room temperature, then incubated with the primary antibodies for at least 5 hours at room temperature (1:100 dilution of the affinity purified antibodies (B1-Af)). After six ten minutes washes with "Net-Gel" (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25 % gelatin and 0.1 % Nonidet P40) at room temperature, immunoblots were incubated for two hours in PBS-3% BSA containing 25 µCi of ^{125}I- protein A (ICN). The immunoblots were then washed as above, dried and autoradiographied using Kodak X- Omat AR film. Relatives protein levels were determined by counting the ^{125}I radioactivity from the nitrocellulose.

For immunoprecipitation after *in vivo* labelling, cells were broken in the presence of glass beads in buffer II (25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 15 mM EGTA, 0.1 mM sodium fluoride, 60 mM β-glycerophosphate, 15 mM para-nitrophenylphosphate, 0.1 mM sodium orthovanadate and 0.1% Triton X100) containing the same inhibitors of proteases as in buffer I. The extracts were boiled 1 minute in the presence of 1% SDS then diluted 10 fold with cold buffer containing DNase I. The soluble protein fraction was recovered by centrifugation for 10 minutes at 11, 000g. After a 30 minute preincubation with protein A-agarose (Pierce) and a 10 min centrifugation at 11, 000g, immunoprecipitations were carried out by incubating the lysate with antibodies overnight at 4°C on a rotator. After centrifugation for 10 minutes at 11, 000g to remove any non-specific precipitate, the immunocomplexes were brought down by further 30 min incubation with 30 µl of protein A-agarose (Pierce) and a low speed centrifugation. The pellets were then washed three times in the following buffer (50 mM Tris-Hcl pH 7.4, 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.1 mM sodium orthovanadate and 0.1 % Triton X100). The final pellets were resuspended in Laemmli sample buffer, heated at 100°C for 3 minutes and subjected to electrophoresis.

Acknowledgments

Jim Duffy, Dave Greene and Phil Renna are acknowledged for artistic assistance. This work was supported by NIH GM34607 to D. B., GM39620 to D. B. and G. D and a Long Island Biological Association Fellowship to B. D.

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