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# *Drosophila* mutants in the 55 kDa regulatory subunit of protein phosphatase 2A show strongly reduced ability to dephosphorylate substrates of p34$^{cdc2}$

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## SUMMARY

**The 55 kDa regulatory subunit of *Drosophila* protein phosphatase 2A is located in the cytoplasm at all cell cycle stages, by the criterion of immunofluorescence. We are unable to detect significant change in protein phosphatase activity during the nuclear division cycle of syncytial embryos. However, cell cycle function of the enzyme is suggested by the mitotic defects exhibited by two *Drosophila* mutants, *aar$^1$* and *twins$^P$*, defective in the gene encoding the 55 kDa subunit. The reduced levels of the 55 kDa subunit correlate with the loss of protein phosphatase 2A-like, okadaic acid-sensitive phosphatase activity of brain extracts against caldesmon and histone H1 phosphorylated by p34$^{cdc2}$/cyclin B kinase, but not against phosphorylase *a*. Thus the mitotic defects of *aar$^1$* and *twins$^P$* are likely to result from the lack of dephosphorylation of specific substrates by protein phosphatase 2A.**

Key words: mitosis, immunolocalization, cdc2

## INTRODUCTION

Considerable attention has been focused upon the role of protein phosphorylation in regulating progression through the cell cycle. This has been concentrated upon the function of protein kinases, especially p34$^{cdc2}$, which regulates the $G_2$-M transition. It is now realized that, in vertebrates, p34$^{cdc2}$ is just one member of a family of related protein kinases, cdks, that form complexes with members of a family of regulatory subunits, the cyclins, to control progression through all cell cycle stages (reviewed by Norbury and Nurse, 1992; Reed, 1992). The p34$^{cdc2}$ kinase is itself regulated by its phosphorylation state. It is activated by phosphorylation of threonine 161 by an enzyme identified in *Xenopus* as p40$^{MO15}$ kinase (Poon et al., 1993; Solomon et al., 1993; Fesquet et al., 1993), and by dephosphorylation of threonine 14 and tyrosine 15 by the cdc25 protein phosphatase (reviewed by Millar and Russell, 1992). Furthermore, cdc25 is activated by phosphorylation that can be mediated by p34$^{cdc2}$ kinase (Hoffmann et al., 1993). It is a somewhat controversial question as to whether protein phosphatase 1 (PP1) or protein phosphatase 2A (PP2A) opposes the kinases that activate either p34$^{cdc2}$ or cdc25 (Lee et al., 1991; Lorca et al., 1992; Clarke et al., 1993), although genetic studies with yeast and the use of phosphatase inhibitors indicate an inhibitory effect of PP2A upon the entry into mitosis (Goris et al., 1989; Felix et al., 1990; Kinoshita et al., 1990; Solomon et al., 1990; Jessus et al., 1991; Lee et al., 1991). The roles of phosphatases in the regulation of other cdks at other stages of the cell cycle, or in dephosphorylating their substrates, are even less clear. However, analysis of SV40 DNA replication in vitro has revealed an S-phase-specific PP2A activity (Virshup and Kelley, 1989; Virshup et al., 1989, 1992; Scheidtmann et al., 1991). Treatment of mammalian cells with low doses of okadaic acid (Vandree and Wills, 1992) and studies with *Drosophila* mutants (Mayer-Jaekel et al., 1993) point towards a function during late mitosis. How the enzyme can exert these specific effects, and how events are regulated in vivo, are still unclear.

It is now apparent that PP2A is a family of holoenzymes. These all have a common core consisting of a catalytic subunit and a 65 kDa regulatory subunit. This core dimer can be further associated with a third, variable subunit of 54 kDa, 55 kDa (PR55), 72 kDa, 74 kDa or 130 kDa (reviewed by Cohen, 1989; Shenolikar and Nairn, 1991; Mayer-Jaekel and Hemmings, 1993). In vitro, the regulatory subunits exert a negative effect on the activity of the catalytic subunit towards most substrates (Kamibayashi et al., 1992). However, the PR55 regulatory subunit (of protein phosphatase 2A) appears to have a positive effect on the dephosphorylation of substrates phosphorylated at S/TP sequences by a cyclin-dependent protein kinase or a mitogen-activated protein kinase (Sola et al., 1991; Agostinis et al., 1992; Goedert et al., 1992; Ferrigno et al., 1993). How far this applies in vivo and whether a similar specificity exists for other holoenzymes, is still an open question.

Two *Drosophila* mutants have been identified with P-element insertions within the gene encoding the PR55 subunit of PP2A. The late pupal/early adult lethal $aar^1$ allele was initially isolated due to mitotic abnormalities in larval brains (Gomes et al., 1993) and subsequently found to coincide with the PR55 locus cloned through its homology with the mammalian gene (Mayer-Jaekel et al., 1993). The mutants have overcondensed chromosomes and abnormal anaphase figures. The second allele, termed $twins^P$, was isolated in a screen for imaginal disc abnormalities and is also lethal at the pupal stage (Uemura et al., 1993). These mutants have a partial pattern duplication of the wing imaginal discs. $aar^1$ and $twins^P$ have different P-elements integrated at exactly the same position within the PR55 gene, four base pairs downstream of the exon IV/intron 4 boundary. A third allele, termed $aar^2$, is female sterile. $aar^1/aar^2$ heterozygotes show similar mitotic abnormalities to $aar^1$ in syncytial embryos.

In this paper we describe the protein phosphatase activity of wild-type and mutant brain extracts towards different substrates. The results provide evidence that the PR55 subunit plays an essential role in directing substrate specificity of PP2A.

## MATERIALS AND METHODS

### Observation of mitotic phenotypes in brains

Analysis of brain squash preparations and whole-mount brains was carried out as described by Gonzales et al. (1991) and Axton et al. (1990), respectively.

### Bacterial expression of recombinant PR55 and antisera purification

Full-length DPR55-1 protein (Mayer-Jaekel et al., 1993) tagged with an amino-terminal polyhistidine metal-binding domain was expressed using the pRSETA bacterial expression vector (Invitrogen, San Diego, CA, USA). An *Nhe*I site was created at the start codon of the original cDNA (Mayer-Jaekel et al., 1993) by the polymerase chain reaction using an oligonucleotide ATGGCTAGC*ATG*GGTCGCTGGG together with a T7 promoter-specific primer. The polymerase chain reaction was performed for 33 cycles with 1 minute denaturation at 92°C, 1 minute annealing (3 cycles 40°C and 30 cycles 64°C), and 2 minutes extension at 72°C. The polymerase chain reaction products were subcloned as *Nhe*I/*Hin*dIII fragments in pRSETA.

Recombinant proteins were expressed in freshly transformed BL21 (DE3) cells induced at an $A_{600}$ of 0.3-0.5 with 2 mM isopropyl-thio-β-D-galactoside for 4 hours. Inclusion bodies were prepared as described by Hendrix et al. (1993). The PR55 protein was purified by SDS-polyacrylamide gel electrophoresis and electroeluted from the gel before injection (200-500 μg per boost).

Antisera were purified using recombinant protein bound to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour with 5% (w/v) skimmed milk in PBS (140 mM NaCl, 2.5 mM KCl, 1.5 mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$, pH 7.0) and subsequently incubated with 1/10 dilution of the antisera in blocking buffer for 14 hours at 4°C. After washing 5 times for 15 minutes each, with PBS containing 1% Tween-20, the antisera were eluted in two washes of 5-15 minutes using 50 mM glycine-HCl, pH 2.3, 500 mM NaCl, 0.5% (v/v) Tween-20, 100 μg/ml BSA, 0.1% sodium azide, and neutralized immediately with 1 M $Na_2HPO_4$. Antisera were used as 1:100 dilutions of the original serum.

The antiserum specific for the catalytic subunit was directed towards the conserved carboxy terminus of the mammalian PP2A catalytic subunit (Shiomi et al., 1994). Similar results were obtained with a second antiserum against this peptide, and with an affinity-purified serum against an internal catalytic subunit peptide (both supplied by B. Favre, Friedrich Miescher-Institut, Basel).

### Western blot analysis and immunolocalization

Brain extracts were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon membranes. After blocking for 30 minutes in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% (w/v) Triton X-100, 0.1% (w/v) Tween-20, 5% (w/v) milk powder, the blots were incubated for 90 minutes with anti-catalytic subunit antisera (1:100) or anti-PR55 antisera (1:100) in blocking buffer, followed by 3× 10 minute washes in the same buffer. The primary antibodies were detected using $^{125}$I-labeled donkey anti-rabbit IgG (Amersham, Buckinghamshire, Great Britain). After a 10 minute wash in blocking buffer, and 3× 10 minute washes without milk powder the blots were exposed on X-OMAT AR films (Kodak, Zürich, Switzerland). For quantification the membranes were exposed to a PhosphorImaging screen, scanned in a PhosphorImager and analyzed using ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA).

Immunolocalization in *Drosophila* embryos was performed as described by Whitfield et al. (1990) and in larval brains as described by Axton et al. (1990) using fluorescein (FITC)-conjugated goat anti-rabbit IgG (Jackson Immune Research Lab, West Grove, PA, USA) as second antibody.

### Protein phosphatase substrates and assays

$^{32}$P-labeled phosphorylase *a* (0.7-1 mole phosphate per mole protein), caldesmon (0.7-1.3 mole phosphate per mole protein) and histone H1 (3.7-6 mole phosphate per mole protein) were prepared as described by Ferrigno et al. (1993), except that the cyclin-dependent kinase was affinity-purified on glutathione-agarose using a GST-cyclin B fusion protein (Pfaffer et al., 1991). The substrates were used at final concentrations in the assay of: 2 μM for caldesmon, 10 μM for histone H1 and 10 μM for phosphorylase *a*. Since the extent of histone H1 phosphorylation also affected the specificity of PP2A towards this substrate the extent of labeling is indicated in each experiment.

The heptapeptide LRRASVA (0.25 mM; Bachem, Bubendorf, Switzerland), corresponding to the phosphorylation site of pyruvate kinase, was phosphorylated by bovine heart protein kinase A (10 units/ml) in 50 mM 4-morpholinoethanesulfonic acid, pH 7, 5 mM magnesium acetate, 0.2 mg/ml fatty acid-free BSA, 375 μM ATP (containing approximately 1.5 GBq [γ-$^{32}$P]ATP per mmole ATP) (Amersham, Buckinghamshire, UK) for 2 hours at 30°C. The reaction was stopped by adding acetic acid to a final concentration of 30% (v/v) and the free ATP was removed by passing the peptide over an anion-exchange AG 1×8 column (Bio-Rad, Glattbrugg, Switzerland) equilibrated in 30% acetic acid. The fractions containing the [$^{32}$P]peptide were lyophilized, resuspended in distilled water and twice relyophilized before dissolving in 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The [$^{32}$P]peptide concentration was adjusted to 180 μM in the same buffer to give a final concentration of 60 μM in the assay.

The protein phosphatase activity was assayed in 30 μl reactions containing 10 μl substrate, 10 μl 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mg/ml BSA, 50 mM 2-mercaptoethanol, and 10 μl cell extract. The reactions were started by substrate addition and stopped after 3-30 minutes at 30°C. Assays with protein substrates were stopped by the addition of 100 μl 20% (w/v) trichloroacetic acid, kept for 10 minutes on ice, and centrifuged for 10 minutes in a microcentrifuge. For phosphorylase *a*, 100 μl of supernatant was used for scintillation counting. For caldesmon and histone H1, 100 μl of supernatant was further processed as described for the peptide substrate. The released $^{32}$P in these reactions was determined as described by Shacter (1984). The phospho-peptide reaction was stopped by the addition of 467 μl 5 mM silicotungstate in 1 mM $H_2SO_4$. After addition of 93 μl (w/v) ammonium (hepta)molybdate in 2 M $H_2SO_4$ and 700 μl of a 1:1 (v/v) solution of isobutanol/toluol the tubes were

mixed vigorously for 10 seconds and centrifuged for 4 minutes. A 500 μl sample of the organic phase containing the free $P_i$ complexed with ammonium molybdate was analyzed by liquid scintillation counting. One protein phosphatase activity unit corresponds to 1 μmol $^{32}P$ released per minute.

### Protein phosphatase activity analysis in synchronized embryos and larval brains

*Drosophila* embryos were synchronized at the entry into mitosis 12 by observing the cortical bud cycles of single embryos as described by Fenton and Glover (1993). The embryos were observed in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.05% Triton X-100, 1 mg/ml aprotinin, leupeptin and pepstatin A) and frozen immediately after lysis on dry ice. Protein phosphatase activity towards different substrates was determined in the crude cell lysates after removing the particulate fraction by centrifugation. In different experiments the extract of a single embryo was diluted and used for three to four protein phosphatase assays with different substrates and inhibitors.

Brains from crawling third instar larvae were dissected in 0.7% NaCl and frozen in test tubes prechilled with dry ice. A total of 10-20 brains were lysed in 100-160 μl extraction buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.05% Tween-20, 1 mg/ml aprotinin, leupeptin and pepstatin A) by 20 strokes with a mini-homogenizer. After 10 minutes of centrifugation in a microcentrifuge the supernatants were diluted to similar protein concentrations (0.15-0.18 mg/ml) and 10 μl samples were used for protein phosphatase assays.

## RESULTS

### Phenotypes of *twins$^P$* and *aar$^1$*

Mutant alleles of the gene encoding the 55 kDa regulatory subunit of PP2A were originally identified in screens for quite different phenotypes: the *twins$^P$* allele was isolated as a developmental mutant that shows pattern duplication of wing discs (Uemura et al., 1993), and the *aar$^1$* allele was isolated as a mitotic mutant (Gomes et al., 1993). We have examined mitotic chromosomes in squashed preparations of brains from homozygous *twins$^P$* and *aar$^1$/twins$^P$* larvae, and found similar mitotic defects to those previously described in *aar$^1$* (Gomes et al., 1993; Mayer-Jaekel et al., 1993). These include overcondensation of chromosomes, and the presence of chromosome bridges or lagging chromosomes at anaphase (data not shown). We also examined the morphology of wing discs in *aar$^1$*. When placed over the deficiency *Df(3R)by62* (Kemphues et al., 1983) that covers the PR55 gene, *aar$^1$* shows the abnormal morphology of wing discs as seen in *twins$^P$*. However, homozygous *aar$^1$* larvae rarely show such abnormalities.

The lethal phases and morphological phenotypes of allelic combinations of *twins$^P$* and *aar$^1$* are summarized in Table 1. Taken together, these observations indicate: firstly, that *twins$^P$* is the stronger allele, as it results in earlier lethality than *aar$^1$* over either *Df(3R)by62* or *twins$^P$*; and secondly, it would seem, that the *twins$^P$* allele is not a null, since *aar$^1$/twins$^P$* shows a later lethal phase than *aar$^1$/Df(3R)by62* and also because the *twins$^P$/Df(3R)by62* results in smaller discs than the *twins$^P$* homozygote. Furthermore, some *aar$^1$* flies survive to adulthood as homozygotes, or when placed over either *twins$^P$* or *Df(3R)by62*. These pharate adults show bristle duplications as seen in weak alleles induced from *twins$^P$* by P-element remobilization (Shiomi et al., 1994).

**Table 1. Lethal phases and morphological phenotypes of *twins$^P$* and *aar$^1$***

| | *Df(3R)by62** | *twins$^P$* | *aar$^1$* |
|---|---|---|---|
| *twins$^P$* | Early pupal (small discs) | Early pupal (duplicated discs) | |
| *aar$^1$* | Early pupal (duplicated discs)/pharate adult (10%) (roughened eyes, duplicated bristles) | Pharate adult/ early adult (duplicated bristles) | Pharate adult/ early adult (duplicated bristles) |

Disc morphologies are that of wing discs in 3rd instar larvae. (From Uemura et al. (1993), Mayer-Jaekel et al. (1993) and this work.)
*Deficiency covering the PR55 locus (Kemphues et al., 1983).

The mitotic and morphological phenotypes of both alleles are rescued by the wild-type PR55 gene in germ-line transformants, confirming that these phenotypes were caused by mutations in the PR55 gene.

### PR55 mutants have low phosphatase activity towards p34$^{cdc2}$ substrates

In common with many mitotic mutants of *Drosophila*, the lethal phase of PR55 mutant alleles suggests that maternally provided protein is sufficient until the late larval stage of development. We wished to determine the effect of the *twins$^P$* and *aar$^1$* mutations on both protein levels and phosphatase activity, and therefore chose to examine brains from third instar larvae, in which the maternal contribution might be expected to be depleted. Western blotting experiments revealed equivalent levels of the catalytic subunit in the two mutant strains relative to wild-type larvae (Fig. 1). However, the amount of PR55 protein is strongly reduced in *aar$^1$* brains (approximately 10% of wild-type) and is barely detectable in *twins$^P$* brains, consistent with *twins$^P$* being the stronger allele.

We then sought to determine the extent to which this reduction in levels of the PR55 subunit affected protein phosphatase activity against several different substrates. In the first set of experiments, we assayed total protein phosphatase activity in extracts of wild-type or *twins$^P$* mutant brains (Table 2). These extracts showed little difference in their ability to dephosphorylate phosphorylase *a*, a substrate for the two major phosphatases type 1 and type 2A. A peptide phosphorylated by protein kinase A has been found to be a more efficient substrate for PP2A holoenzymes in comparison to PP1 (R. M.-J., unpublished data). When this is used as a substrate, the mutant extract shows a 29% reduction in total activity. However, when either histone H1 or caldesmon phosphorylated by p34$^{cdc2}$/cyclin B was used as substrate, the phosphatase activity in the *twins$^P$* brain extract was found to be only 27% and 11%, respectively, of wild-type activity.

In order to determine whether this reduction in phosphatase activity against certain substrates reflected the activity of a type 2A-related phosphatase, we repeated these assays in the presence and absence of okadaic acid, and extended the study to examine *aar$^1$* mutant brains (Fig. 2). The most dramatic reduction in okadaic acid-sensitive phosphatase activity was again found using as a substrate histone H1 or caldesmon phosphorylated by p34$^{cdc2}$/cyclin B. In both cases, the activity in

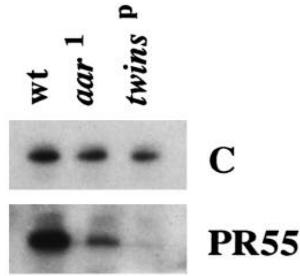


**Fig. 1.** PR55 protein levels in wild-type and mutant brains. Cell extracts of third instar larval brains from wild type (wt), *aar*[1] or *twins*[P] (5 µg each) were analyzed by western blotting with anti-PR55-1 antiserum or with serum directed against the carboxy-terminal peptide of the catalytic subunit (Shiomi et al., 1994), which is conserved between *Drosophila* and mammals.

**Table 2. Total protein phosphatase activity in extracts of wild-type and *twins*[P] brains**

| | Wild-type (mU) | *twins*[P] (mU) | % of wild-type activity |
|---|---|---|---|
| Phosphorylase *a* | 13.2 | 13.8 | 105 |
| Peptide | 7.74 | 5.5 | 71 |
| Histone H1 | 1.99 | 0.51 | 26 |
| Caldesmon | 1.43 | 0.15 | 11 |

Protein phosphatase activity towards the indicated substrates was determined in cell-free extracts from 10 wild-type or *twins*[P] brains. Histone H1 was labeled to 3.7 mol phosphate per mol protein.

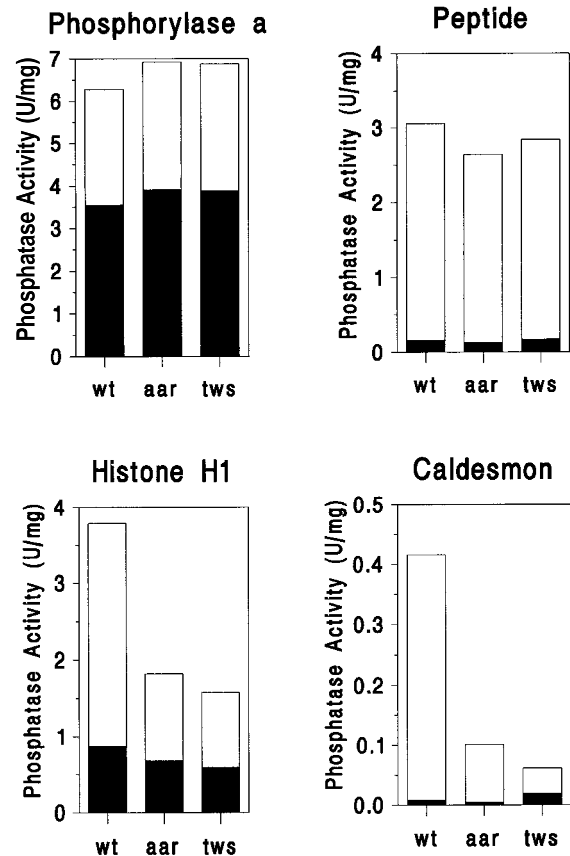


**Fig. 2.** Protein phosphatase activity (U, units) in wild-type (wt) and mutant (*aar*[1], *twins*[P]) brains. Protein phosphatase activity towards the indicated substrates was determined in larval brain extracts in the absence (open bars) or presence (filled bars) of 10 nM okadaic acid. The $p34^{cdc2}$ phosphorylated histone H1 was phosphorylated to 6 mol phosphate per mol protein and used at 10 µM final concentration in the assay.

*twins*[P] brains was lower than in *aar*[1] brains, consistent with the relative strengths of these mutant alleles. The reduction in activity was the most pronounced using caldesmon, where *twins*[P] brains displayed only 14% of the wild-type phosphatase activity. The activity of the mutant extracts towards histone H1 was dependent upon the degree of phosphorylation of this protein. In the experiment shown in Table 2, in which the *twins*[P] mutant extract showed a 73% reduction in activity compared with wild-type, the histone H1 was phosphorylated to the extent of 3.7 moles phosphate per mole protein. In the experiment reported in Fig. 2, the substrate was hyperphosphorylated (6 moles phosphate per mole protein). The less extensive reduction in dephosphorylation (58%) measured in the second experiment is likely to be a consequence of this hyperphosphorylation (see Discussion).

## Localization of PR55 protein in the cell cycle

Regulatory subunits of type 1 protein phosphatase are known to have a role in targeting the catalytic subunit to its substrates (Hubbard and Cohen, 1993). We therefore wondered whether the PR55 protein might be associated with a particular structure in the cell, and if so, whether its localization might change during the cell cycle. *Drosophila* shows different types of cell cycles in its development. In syncytial embryos, the division cycles are very rapid and comprise alternating S and M phases, without gap phases. A $G_2$ phase is introduced during cycle 14 at the time of cellularization, and a $G_1$ phase not until cycle 17. In the later stages of embryogenesis and in larval development, most cells leave the division cycle to become polytenized, with the exception of some cells in the central nervous system and imaginal cells, which continue to proliferate in mitotic cycles with two gap phases. We examined the localization of PR55 protein in these three types of mitotic cycles by immunostaining using anti-PR55 antisera.

The distribution of the PR55 subunit in syncytial embryos at blastoderm is shown in Fig. 3A,B. The cytoplasmic region surrounding the cortical layer of nuclei was stained uniformly, whereas the region occupied by chromatin (corresponding to that contained by the nuclear or spindle envelopes) showed much weaker staining throughout the cell cycle. The pole cells, which divide out of synchrony with the syncytium, show a similar staining pattern. In cellularized cycle 14 embryos (Fig. 3C), the PR55 subunit appears to be located throughout the cytoplasm of interphase cells, and throughout the whole cell as it progresses through mitosis. Fig. 3D shows several giant neuroblasts in different stages of the mitotic cycle in a whole-mount preparation of the brain of a third instar larva. The main staining is seen in the cytoplasmic region of both interphase and mitotic cells. In *twins*[P] mutant brains, only a very weak signal was detected and no preferential staining of cytoplasm was observed, confirming the specificity of this antisera in immunostaining experiments (data not shown). Consequently, we conclude that if PR55 protein does show any specific local-

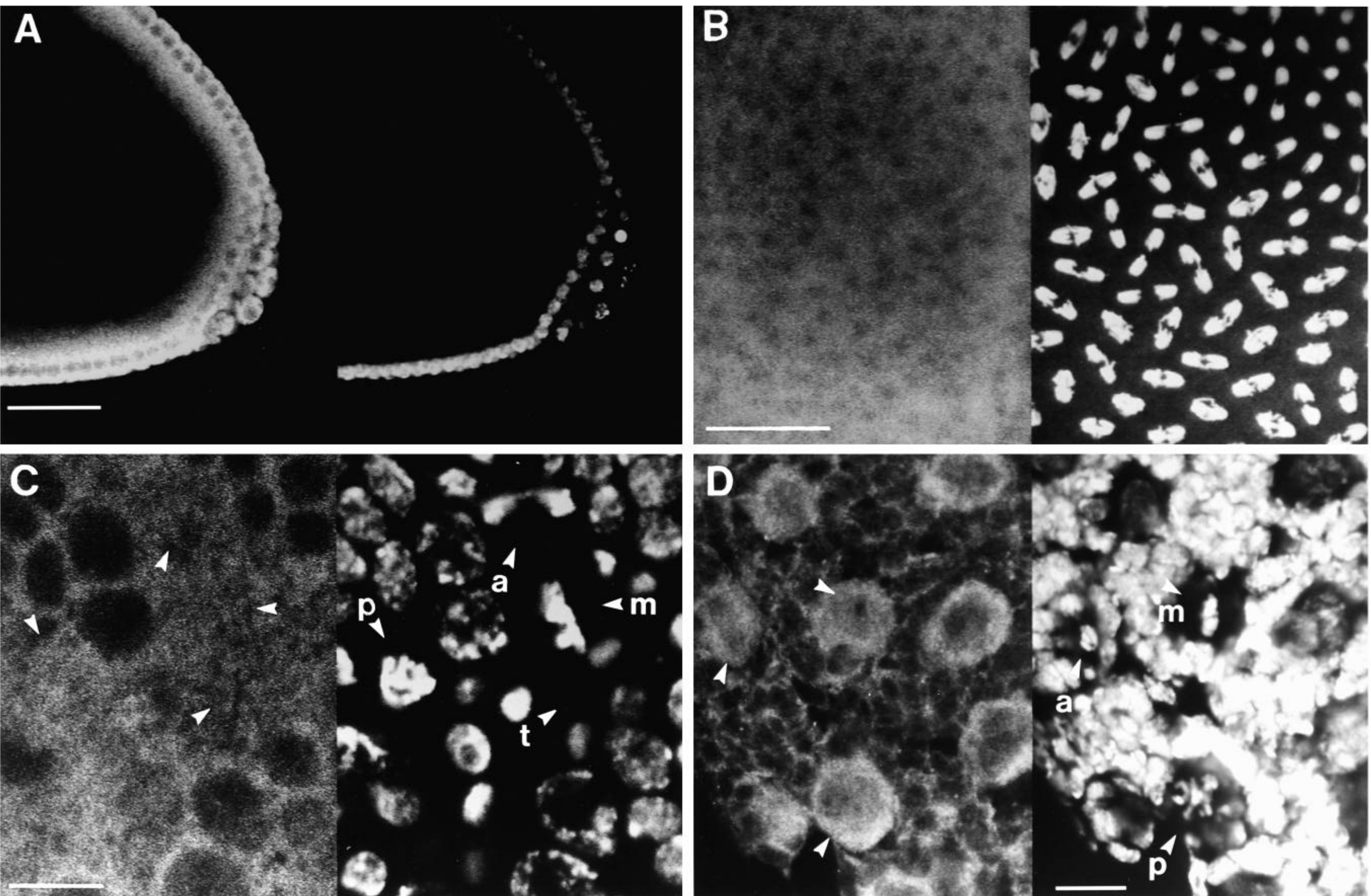


**Fig. 3.** Immunolocalization of PR55. The micrographs show the immunolocalization of the PR55 subunit on the left and the propidium iodide staining of DNA on the right. (A) The posterior pole of a syncytial embryo with the interphase nuclei in the periphery. (B) A syncytial embryo in anaphase. (C) A cellularized embryo in late cycle 14. (D) The central anterior part of a brain ganglion. The large cells are the proliferating neuroblasts. Individual mitotic cells in C and D are indicated by arrowheads and labelled: p, prophase; m, metaphase; a, anaphase; t, telophase. Bars: 10 µm in A and B; 50 µm in C and D.

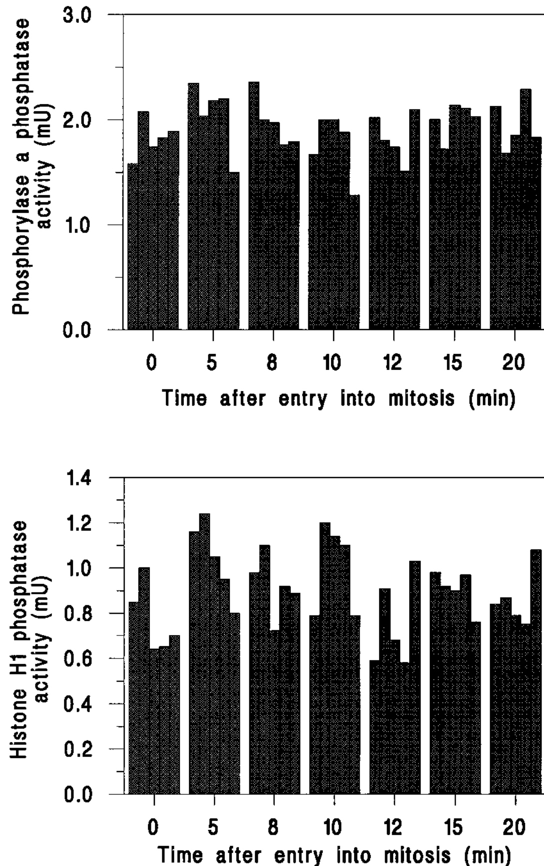


**Fig. 4.** Protein phosphatase activity in cortical bud cycles. Five embryos were analyzed for each time point after cortical bud breakdown. Phosphorylase *a* or histone H1 (3.7 mol phosphate per mol protein) phosphatase activity was determined in the same extracts.

ization within the cytoplasm, it is beyond the resolution of these experiments. Analysis of the subcellular localization of the catalytic and PR65 subunits in embryos and imaginal discs showed the same cytoplasmic localization as the PR55 subunit (data not shown).

## Phosphatase activity during the mitotic cycles of syncytial embryos

Fenton and Glover (1993) recently described a strategy for assaying the cyclical activity of p34$^{cdc2}$/cyclin B kinase and polo kinase in extracts of individual *Drosophila* syncytial embryos by following their cortical budding cycles. In order to determine whether PP2A activity varies throughout these rapid nuclear division cycles, we analyzed protein phosphatase activity towards different substrates following the synchronous entry of individual embryos into mitotic cycle 12. Such embryos progress through mitosis from prophase at the start of the time-course to telophase 10-12 minutes later. We were unable to observe any significant change in protein phosphatase activity throughout this time course, using either phosphorylase *a* as a general substrate, or p34$^{cdc2}$/cyclin B-phosphorylated histone H1 as a preferred substrate for the PR55 containing PP2A holoenzyme (Fig. 4). Similar results were obtained using either p34$^{cdc2}$ phosphorylated caldesmon or the peptide substrate (data not shown).

# DISCUSSION

*Drosophila* mutants with reduced levels of the PR55 subunit show pleiotropic phenotypes. The three mutant alleles that have been examined show mitotic abnormalities in anaphase. Additional defects are also seen in cell fate determination. The strong allele, *twins*$^{P}$, shows pattern duplication in the wing imaginal discs, and weaker alleles show a bristle duplication (Shiomi et al., 1994), suggesting a specific role in these processes. As none of the alleles are nulls, it is possible that there may be additional functions provided by very low levels of PR55, or indeed by other PP2A holoenzymes. However, the extent of the mutant phenotype does appear to reflect the level of expression of the PR55 protein. Reduction of levels of protein in *aar*$^{1}$ leads to mitotic defects in larval brain cells, but almost complete loss of protein is required to see both the mitotic defects and the wing disc abnormalities in *twins*$^{P}$.

The reduction in PP2A activity in extracts of mutant brains is also in agreement with the relative strengths of the mutant alleles when assayed against preferred substrates phosphorylated by p34$^{cdc2}$/cyclin B. The most striking effect of the mutations is to change the substrate specificity of protein phosphatase activity in the extracts. The limited reduction in the PP2A-like activity towards the phospho-peptide shows that only part of the activity observed in the wild-type is due to the trimeric PP2A holoenzyme containing the PR55 subunit. The remaining activity is most likely derived from the other PP2A holoenzymes, and probably also from related protein phosphatases that are not yet enzymically characterized (Chen et al., 1992). The proline residue located at the carboxy-terminal side of p34$^{cdc2}$ phosphorylation sites (Chambers and Langan, 1990) is primarily a negative determinant for the dephosphorylation by PP2A catalytic subunit as determined using phospho-peptide substrates (Pinna et al., 1989; Donella-Deana et al., 1990). However, the PR55 subunit appears to overcome this inhibition, making the p34$^{cdc2}$ site a preferred substrate for this holoenzyme (Sola et al., 1991; Goedert et al., 1992; Agostinis et al., 1992; Ferrigno et al., 1993). Comparative analysis of purified PP2A holoenzymes as well as the analysis of Mono Q fast protein liquid chromatography profiles from *Xenopus* oocytes showed that PP2A activity is mainly due to the trimeric form containing the PR55 subunit (P.F., unpublished data). In the experiments we present in this paper, we used histone H1 preparations that had been phosphorylated to varying extents, and found that phosphatase activity towards a hyperphosphorylated substrate was not as extensively reduced in the mutant extracts. The hyperphosphorylation is likely to have involved sites that are not classical p34$^{cdc2}$ sites, and therefore not recognized as efficiently by the PR55-containing PP2A holoenzyme. This is supported by the fact that there is a considerable amount of okadaic acid-insensitive activity in embryo extracts, which on the basis of the analysis of Mono Q profiles is PP1 (R.M.-J. and N.A., unpublished data).

Of the p34$^{cdc2}$ phosphorylated proteins used as substrates in our in vitro assays, histone H1 may not be a significant substrate in vivo as the PR55 protein appears to be excluded from the nucleus or the vicinity of chromatin at most cell cycle stages. This is not to say, however, that a small fraction of the protein could not have a nuclear distribution. Histone H1 is a good model substrate for PP2A, since in addition to its ability to be specifically phosphorylated by p34$^{cdc2}$ its polycationic

nature leads to a stimulation of PP2A and an inhibition of PP1 activity (Pelech and Cohen, 1985). p34$^{cdc2}$ phosphorylated caldesmon, which appeared to be the most specific substrate for the trimeric PP2A in our experiments, could well be a substrate for the enzyme in vivo. Caldesmon interacts with microfilaments during interphase, but dissociates again in mitosis as a consequence of its phosphorylation (Yamashiro et al., 1990). Thus, the dephosphorylation of caldesmon at the end of mitosis might play a role in restoring the interphase state of the microfilament network.

What are the likely roles for the PR55-containing PP2A enzyme in vivo? The cell cycle defects observed in PR55 mutants indicate possible involvement in a wide variety of events. There are affects on spindle microtubule organization and on the extent of chromosome condensation, anaphase defects that could indicate defects in DNA replication or decatenation of chromatids, and defective interactions between spindle and kinetochore. Our observations indicate that there are no dramatic changes in either the sub-cellular distribution of PR55, or the overall phosphatase activity directed against p34$^{cdc2}$ substrates at different stages of the cell cycle. However, subtle localized changes in distribution or activity would not necessarily be detected by the methods used. Furthermore, it is also difficult to exclude the possibility that the phosphatase activity changes in the cell lysates after lysis of the nucleus, since for starfish oocytes (but not *Xenopus*) there is a report about a nuclear component inhibiting PP2A activity (Picard et al., 1991). Among the possible targets for the PR55-containing PP2A holoenzyme could be enzymes involved in cell cycle regulation, including kinases or phosphatases that are themselves regulated by reversible phosphorylation. The dual-specificity phosphatase, cdc25, or its homologues, is required to activate p34$^{cdc2}$ and can also be activated by p34$^{cdc2}$ in a positive autoregulatory loop. Failure of PP2A to dephosphorylate these sites in the PR55 mutants could lead to premature activation of this loop, thus perturbing the timing of entry into mitosis. If this were to occur before DNA replication was fully completed, it would lead to one aspect of the mutant phenotype, the formation of chromatin bridges in anaphase. Alternatively, structural components of the cell that are modified by p34$^{cdc2}$ are potential substrates for this form of PP2A. Moreover, the substrates of the family of cdk enzymes could also be good candidates, given the relatedness of their phosphorylation sites, although as yet one cannot speculate upon a specific cdk/cyclin complex.

Although we show that the 55 kDa regulatory subunit of PP2A directs the specificity of dephosphorylation towards substrates phosphophorylated by p34$^{cdc2}$ in vitro, the determination of the identity of its true in vivo substrates remains a future challenge. The existence of several other regulatory subunits of PP2A suggests that the regulation of its function in the cell cycle may be considerably more subtle than has been revealed by studies using inhibitors that selectively inactivate the catalytic subunit. It is of considerable interest to determine the roles of the other subunits in the PP2A family of holoenzymes, a problem that can be approached by a combination of genetic and biochemical studies.

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