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Studies have indicated that cyclin D protein levels are modulated post-transcriptionally by the ubiquitin-mediated protein degradation pathway. The specific E2 and E3 enzymes postulated to target cyclin D for ubiquitination are the ubiquitin conjugating enzyme, CDC34, and the ubiquitin protein ligase called SCF/ring (Skp1, Cullin , F-box, ring protein). Our findings indicate that CDC34 is phosphorylated by Casein Kinase 2 (CK2) on five carboxyl-terminal residues. Mutation of these residues of CDC34 significantly alter the cell localization of CDC34 and potentially its function with the SCF complex. A recent study has shown that the breast cancer cell line, MCF-7, highly over-expresses cyclin D and is significantly reduced for SCF-mediated ubiquitination activity specific for cyclin D. Therefore, we propose CDC34-SCF activity specific for cyclin D is regulated by CDC34 phosphorylation, compartmentalization, and protein-protein interactions. The goal of our studies is to understand the biological significance of CDC34 phosphorylation and its impact in cyclin D protein regulation.

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The role of ubiquitin-mediated proteolysis of cyclin D in breast cancer

INTRODUCTION

Studies have indicated that cyclin D protein levels are modulated post-transcriptionally by the ubiquitin-mediated protein degradation pathway. The specific E2 and E3 enzymes postulated to target cyclin D for ubiquitination are the ubiquitin conjugating enzyme, CDC34, and the ubiquitin protein ligase called SCF/ring (Skp1, Cullin , F-box, ring protein). Our findings indicate that CDC34 is phosphorylated by Casein Kinase 2 (CK2) on five carboxyl-terminal residues. Mutation of these residues of CDC34 significantly alter the cell localization of CDC34 and potentially its function with the SCF complex. A recent study has shown that the breast cancer cell line, MCF-7, highly over-expresses cyclin D and is significantly reduced for SCF-mediated ubiquitination activity specific for cyclin D. Therefore, we propose CDC34-SCF activity specific for cyclin D is regulated by CDC34 phosphorylation, compartmentalization, and protein-protein interactions. The goal of our studies is to understand the biological significance of CDC34 phosphorylation and its impact in cyclin D protein regulation. In an attempt to understand the biological significance of CDC34 phosphorylation and cyclin D proteolysis, we have generated WT CDC34, truncation mutants of CDC34 and phosphomutants of CDC34 in yeast expression plasmids, mammalian expression plasmids and bacterial expression plasmids as well as SCF/ring components in mammalian expression vectors. These reagents have allowed us to examine the biological significance of CDC34 phosphorylation *in vitro* and *in vivo*.

BODY

TASK 1: Months 1-9

To study the regulation of CDC34 function by phosphorylation in normal and transformed breast cells.

a. To study how the phosphorylation of CDC34, primarily by CK2, may regulate the function of CDC34 during the G1/S phase transition.

b. To examine the phosphorylation of CDC34 and cyclin D half life in transformed cells and normal breast cancer cells.

A. To study the biological significance of CDC34 phosphorylation at the G1/S transition, we transiently over expressed wildtype human CDC34 (WT), the non-phosphorylated CDC34 mutant (5 PT MUT), or the carboxyl-terminal 36 amino acid truncation mutant of CDC34 (1-200) into 10T-1/2 cells followed by flow cytometry and cell cycle analysis (**Figure 1** of Appendix). Western analysis of transfected HA-tagged and endogenous CDC34 proteins indicated that the transfected CDC34 was expressed between 5-10 fold over the level of endogenous CDC34 (data not shown). The results suggest that overexpression of the CDC34 WT and 1-200 results in a significant increase in the G1

population and a concomitant decrease in the number of cells both in S and G2/M phases compared to cells transfected with the vector alone (VECTOR) (Fig. 1, top panel). A similar, but less pronounced effect was observed with the CDC34 5 PT MUT (Fig. 1, top panel). In addition to 10T-1/2 cells which are diploid murine fibroblasts, we also transfected U2OS human osteosarcoma cells with vector alone, CDC34 WT, 5 PT MUT, 1-200, and the CDC34 mutant which alters the active site cysteine and downstream leucine to serine (CL-S). In U2OS cancer cells, the effect of CDC34 overexpression results in an increase in G1 cells, a mild decrease in S phase cells for some samples, and a decrease in the number of G2/M cells (Fig. 1, bottom panel). Overall, the effects of CDC34 overexpression were less pronounced in the U2OS cancer cell line. The preliminary results in 10T-1/2 cells have been repeated, but will be further studied. Thus far, the results suggest that overexpression of any CDC34 molecule results in a dominant negative effect on the entry into S and M phases. This implies that the overexpressed CDC34 binds and perhaps titrates out a critical regulator of the cell cycle making it difficult to use this approach to specifically study the CDC34 phospho-mutant. A similar effect of CDC34 ectopic overexpression was observed in Xenopus extracts where both the CDC34 WT and CL-S mutant inhibited the onset of S phase at high concentrations. How the overexpression of CDC34 influences the stability of CDC34 substrates such as p27-Kip1, cyclin D, cyclin E, and Wee1 is under current investigation.

B. We have examined the phosphorylation of endogenous CDC34 in normal cells and several established breast cancer cell lines. We have previously shown that CDC34 immunoprecipitates with associated kinase activity. Therefore, we wanted to determine whether an increase in CK2 activity in cancer cells correlates with an increase in CDC34 phosphorylation and an increase in cyclin D levels. For our studies, we chose normal and breast tumor cell lines that were available at this Institute for our studies. We first measured the level of phosphorylation of endogenous CDC34 by the CDC34-associated kinase (which our studies indicate is CK2) in normal or tumor breast cell lines. We performed an immunoprecipitation of cell lysate with affinity purified CDC34 antibody (CDC34 Ig) or non-immune rabbit antibody (RIg) followed by an in vitro kinase reaction (**Figure 2** of Appendix). However, these studies did not show a consistent increase in CDC34 phosphorylation in breast tumor cells over normal breast cells as we had expected (Fig. 2, arrow indicates CDC34 protein). MCF7 breast cancer cells had already been shown to have an increase in cyclin D1 levels and a decrease in ubiquitination activity, however it is still unknown why these cells have decrease ubiquitination activity for cyclin D1. We did not observe an increase of CDC34 phosphorylation in any other breast cancer cell line tested. In continuing studies, we will examine other established breast cell lines commercially available. We will also examine cells derived of other tissue

types and we will first determine whether CK2 is elevated in the tumor cells before analyzing the phosphorylation status of endogenous CDC34 by orthophosphate labeling and immunoprecipitation.

Task 2: Months 6-24

a. To determine the cell localization of the cyclin D ubiquitinating activity mediated by CDC34-SCF in normal and breast cancer cells

b. To determine the mechanism of CDC34 compartmentalization through protein-protein interactions with known CDC34-interacting proteins and to characterize how phosphorylation may regulate the interaction of CDC34 with other proteins

A. It was shown that phosphorylation by glycogen synthase kinase 3β (GSK-3β) on threonine 286 of cyclin D1 is required ubiquitination, nuclear export and degradation (Diehl et al., 1998). At the time of the submission of this grant, it was not known if ubiquitination of cyclin D1 occurred in the nucleus or cytoplasm. However, the time this grant was awarded it had been published that the ubiquitination activity for cyclin D1 occurs in the nucleus in U20S cells (Ganiatsas et al., 2001). CDC34 is a predominately nuclear protein; however, we have observed a more nuclear pattern when immunostaining endogenous CDC34 in U2OS cells (unpublished observation). It would be proposed that the ubiquitination activity for cyclin D1 would also be nuclear in other tissue types. However, we will examine the cyclin D ubiquitinating activity mediated by CDC34-SCF in fractionated cytoplasmic or nuclear fractions in normal and breast cancer cells as proposed in task 2a in the upcoming year.

B. The study of CDC34 compartmentalization through protein-protein interactions with known CDC34 interacting proteins has lead to a manuscript in preparation. The E2, CDC34, has been shown to function in association with the E3, SCF/ring, to mediate the G1 to S phase transition in budding yeast and mammals. CK2 is an essential enzyme in *S. cerevisiae* as disruption of both α and α' genes together result in lethality (Padmanabha *et al.*, 1990). Human and budding yeast CDC34 proteins share ~40% amino acid identity. The amino-terminal catalytic domain is the most highly conserved region among CDC34 proteins and although little homology is observed in the carboxyl-terminal domains, both human and yeast CDC34 proteins exhibit highly acidic tails. The *CDC34* gene in *S. cerevisiae* is essential and *cdc34* temperature sensitive (ts) mutant strains arrest in G1 at the non-permissive temperature with a phenotype of multiple buds (Hereford and Hartwell, 1974; Schwob *et al.*, 1994). The human CDC34 gene fully complements the growth defect phenotype of *cdc34* ts mutant yeast strains (Plon *et al.*, 1993). In order to determine whether the phosphorylation of sites within the human CDC34 acidic tail may be required for functional complementation of the budding yeast ts strain, *cdc34-2*, we cloned

human CDC34 WT, 1-200, 5 PT MUT (S203A, S222A, S231A, T233A, S236A) and CDC34 S203E, S222E into the yeast expression vector, pSF315B, to perform complementation studies. Previous studies have shown that the active-site cysteine double point mutation of human CDC34 (C93S, L97S) cannot complement the *cdc34-1* ts strain (Yew and Kirschner, unpublished observation). The results show that the human CDC34 active site mutant (C93S, L97S) cannot complement the budding yeast *cdc34-2* ts strain, while the 5 PT MUT hCDC34 and the hCDC34 glutamic acid mutant (hCDC34 S203E ,S222E) proteins appear to complement the ts strain like WT hCDC34 at the non-permissive temperature of 37°C. Interestingly, the human CDC34 1-200 cannot complement the *cdc34-2* ts growth defect, indicating an essential, previously undescribed role for human residues 201 to 236 in the complementation of *cdc34* ts strains **(Figure 3** of Appendix). These results indicate that although the acidic tail domain of human CDC34 is required for the complementation of the *cdc34-2* ts strain, phosphorylation of sites within the tail domain of human CDC34 are not essential for the complementation function in budding yeast. The inability of hCDC34 1-200 to complement the *S. cerevisiae* ts strain is likely to be due to the inability of the hCDC34 1-200 protein to interact with a critical partner in budding yeast. The ubiquitin conjugating enzyme receives an activated ubiquitin molecule through thioesterification from the ubiquitin activating enzyme. Therefore, one trivial explanation for the lack of hCDC34 1-200 to complement the temperature sensitive strain is the in ability of the ubiquitin conjugation enzyme to receive the activated ubiquitin. To test this, we purified 6xhis WT CDC34 and CDC34 mutants and collaborated with Michele Pagano and Joanna Bloom of New York University School of Medicine and they tested the ability of the purified proteins to be charged in a well established in vitro E2 charging reaction (Ganoth et al., 2001). At 60 minutes, human WT CDC34, the truncation mutant CDC34 1-200, the phosphomutant of CDC34 5 pt A, as well as the glutamic acid mutant CDC34 5 pt E were all efficiently charged by the activated ubiquitin. (**Figure 4** of Appendix). This implies that the WT CDC34 and CDC34 mutants were all able to receive a charged ubiquitin from the ubiquitin activating enzyme, E1. Because the tail domain of CDC34 appears to be important for CDC34 function, our collaborators tested the ability of the same purified proteins to ubiquitinate a known substrate of CDC34/SCF complex, p27Kip1, *in vitro*. We found that the WT CDC34 was able to ubiquitinate p27Kip1 in vitro as was the phosphomutant and glutamic acid mutant of CDC34. However, the CDC34 1-200 truncation mutant could not (**Figure 5** of Appendix). The mammalian SCF/ring complex is composed of the F-box binding protein Skp1, the F-box protein Skp2, the scaffold protein Cullin1 (CUL1), and the ring finger protein ROC1/RBX1/Hrt1. The crystal structure of the SCF/ring complex and genetic studies from budding yeast indicate that CDC34 directly associates with the Cullin and the ring finger protein suggesting mammalian CDC34 may directly bind CUL1 and ROC1 (Zheng et al., 2002). We performed studies to determine whether removal of the carboxyl-terminal 36 amino acids of CDC34 would affect the putative binding of CDC34 to CUL1 or ROC1 in vivo. Because we propose that the phosphorylation of CDC34 by CK2 occurs strictly within the acidic carboxyl-terminal tail of CDC34, this

study could help us understand whether the tail domain of CDC34 functions to regulate the binding of CDC34 to components of the SCF/ring. We transiently transfected 293 cells with Flag-tagged full length CDC34 (1-236) or truncation mutants of CDC34 removing the final 36 amino acids (1-200), the final 56 amino acids (1-180), or the final 96 amino acids (1-140) as well as CDC34 5 PT MUT (phosphomutant) and a glutamic acid mutant of CDC34, CDC34 5 pt E. At the same time, HA-tagged CUL1 or ROC1 was co-transfected into cells followed by immunoprecipitation and western analysis on the resulting transfected cell lysates. Our overexpression binding studies indicated that CUL1 and ROC1 both appear to associate with CDC34 within amino acids 1-180 of CDC34 (data not shown). In addition, we observed both CUL1 and ROC1 in the CDC34 5 PT MUT and CDC34 5 PT E immunoprecipitations (data not shown). During these studies, Wu et al., has published that amino acids 194-208 of CDC34 are important for CUL1/ROC1 binding. In addition, amino acids 208-236 are important for efficient CUL1/ROC1 binding. We then examined the requirements for direct CDC34 binding to CUL1 and ROC1 using *in vitro* binding studies. *In vitro* binding studies would determine how the phosphorylation of CDC34 might quantitatively regulate the association or function of the CDC34-SCF/ring complex in vivo as compared to overexpression studies. We therefore performed in vitro co-immunoprecipitation assay by co-in vitro translating CUL1/ROC1 and binding to bacterial purified 6xhis WT hCDC34, 6xhis hCDC34 1-200, 6xhis hCDC34 5 pt A or 6xhis CDC34 5 pt E. Our experiments have indicated that hCDC34 1-200 and hCDC34 5 pt A are deficient in binding CUL1 and ROC1 relative to WT CDC34 whereas hCDC34 5 pt E is statistically like WT hCDC34 (**Figure 6** of Appendix). It is known that proteins with a nuclear localization sequence (NLS) bind importin alpha and are transported to the nucleus. We examined the primary sequence of CDC34 and did not find a canonical nuclear localization sequence (NLS) however, we tested the ability of CDC34 to bind to importin alpha in a co-immunoprecipitation assay. We did not observe any interaction of hCDC34 WT with importin alpha in vitro (**Figure 7** of Appendix). Because we see an altered localization of hCDC34 1-200 and hCDC34 5 pt A in U20S cells and CDC34 does not bind importin alpha, we propose that binding CUL1/ROC1 may be important for CDC34 localization.

KEY RESEARCH ACCOMPLISHMENTS

- Examination of CDC34 phosphorylation in Normal breast cells and Breast Cancer cells
- The mechanism of CDC34 phosphorylation and compartmentalization through protein-protein interactions with known CDC34-interacting proteins.

REPORTABLE OUTCOMES

THE ACIDIC TAIL DOMAIN OF HUMAN CDC34 IS REQUIRED FOR P27KIP1 UBIQUITINATION AND COMPLEMENTATION OF A cdc34-2 TEMPERATURE SENSITIVE STRAIN IN BUDDING YEAST. Block, K., Bloom J., Pagano M., Yew, P.R. Manuscript in preparation

AWARDS:

None

CONCLUSIONS

We have generated WT CDC34, truncation mutants of CDC34 and phosphomutants of CDC34 in yeast expression plasmids, mammalian expression plasmids and bacterial expression plasmids as well as SCF/ring components in mammalian expression vectors. These reagents have allowed us to examine the biological significance of CDC34 phosphorylation *in vitro* and *in vivo*. It has become apparent that overexpression of CDC34 WT causes a dominant negative effect in mammalian cells. Therefore, it will be important to study the mechanisms of CDC34 compartmentalization and its affects of cyclin D ubiquitination by examining endogenous CDC34. We will fractionate nuclear and cytoplasmic cell extracts generated from normal breast cells and breast cancer cell lines and examine cyclin D ubiquitination activity in those cell lines. The cell lines that appear to have increased cyclin D levels and low ubiquitination activity will be furthered studied for CDC34 phosphorylation and localization. These studies will help us determine if CDC34 phosphorylation is directly linked to cyclin D levels and ubiquitination activity.

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FIGURE 1

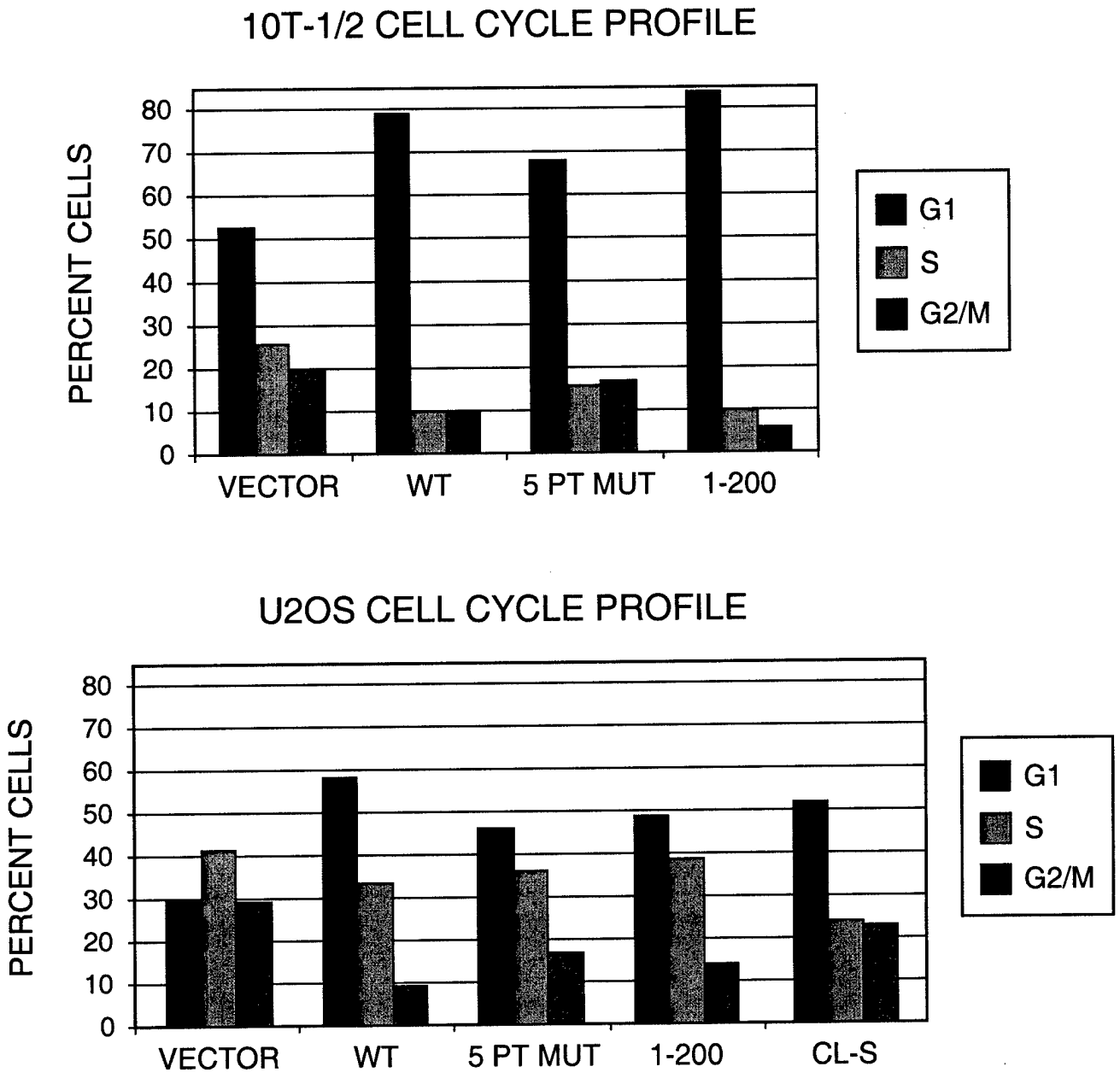
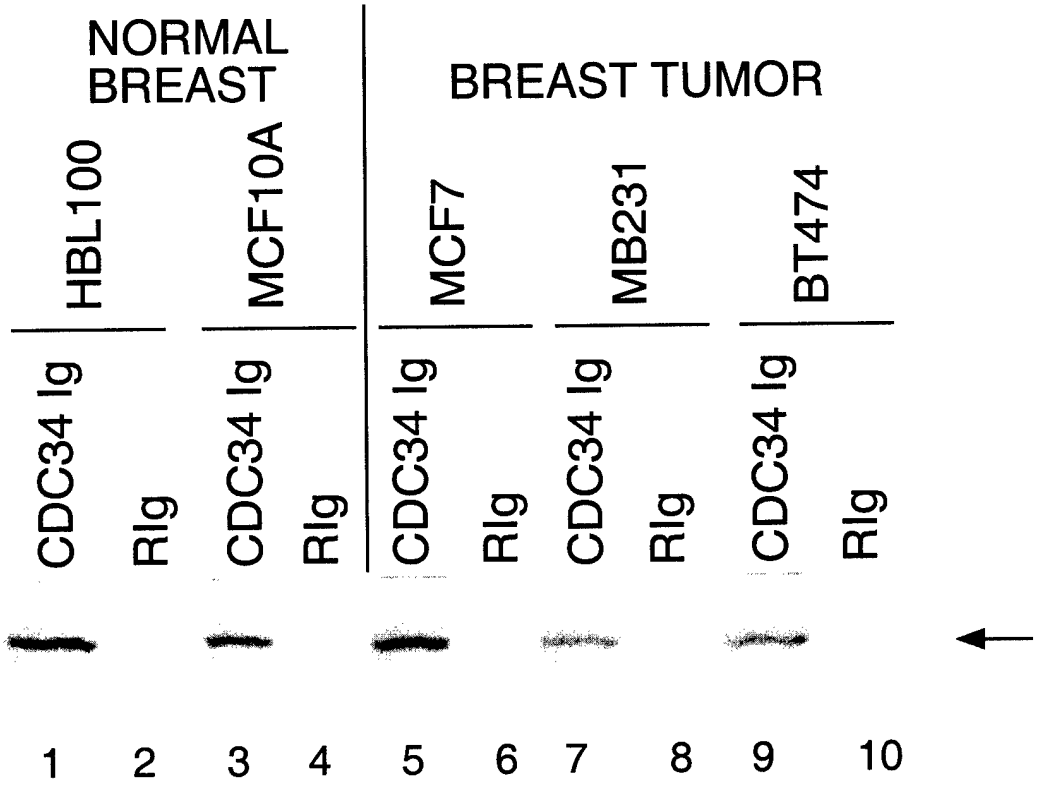


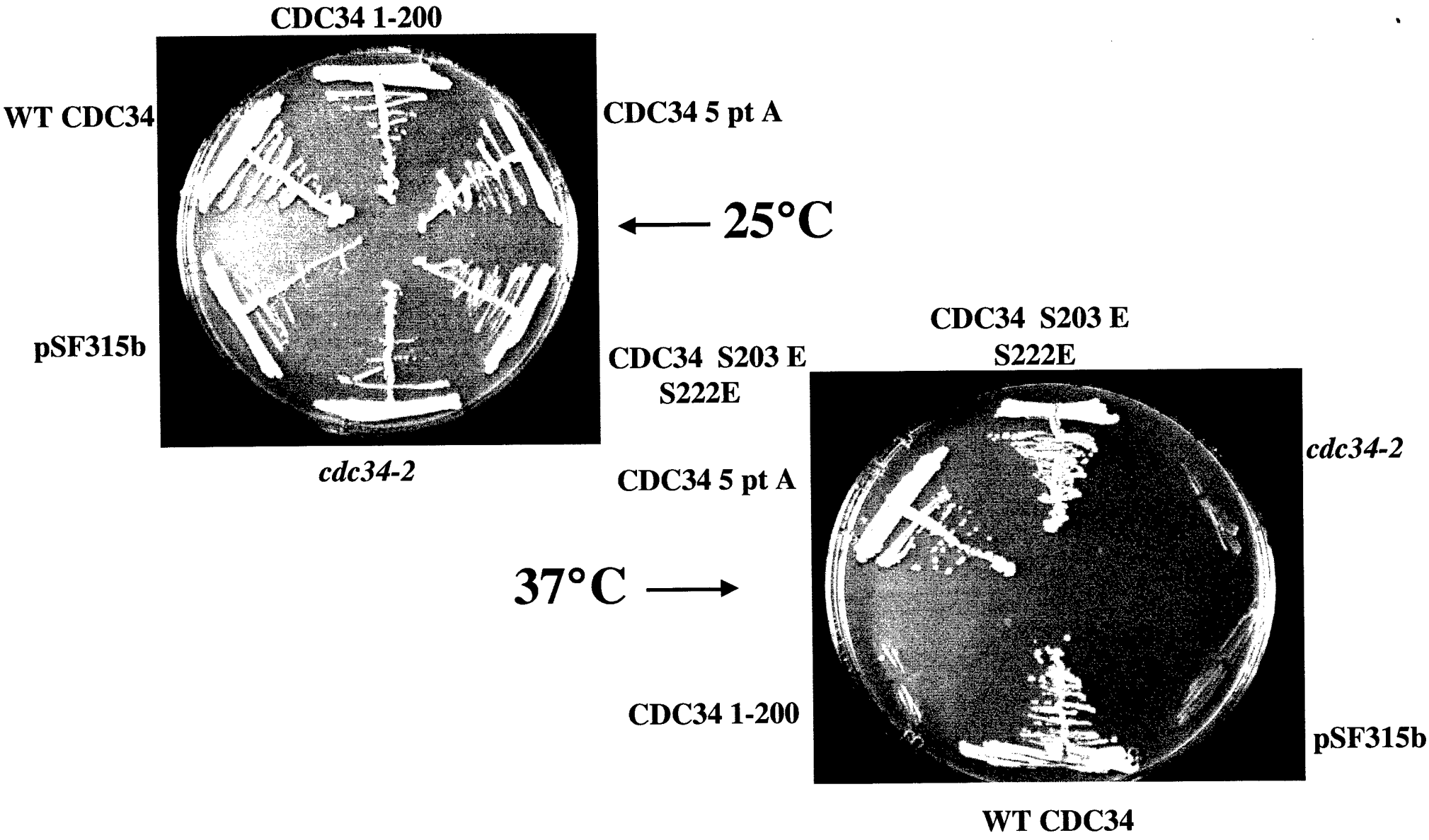
Figure 1. *Transient transfection of CDC34 into mammalian cells causes an increase of cells in G1 phase.* FACS analysis. (Top panel) We transiently overexpressed pCS2+ (VECTOR), wildtype human CDC34 (WT), the nonphosphorylated CDC34 mutant (5 PT MUT), a carboxyl-terminal 36 amino acid truncation mutant of CDC34 (1-200) or a dominant negative CDC34 (CL-S) into 10T1/2 cells followed by flow cytometry and cell cycle analysis or (bottom panel) in osteosarcoma cells, U2OS.

FIGURE 2



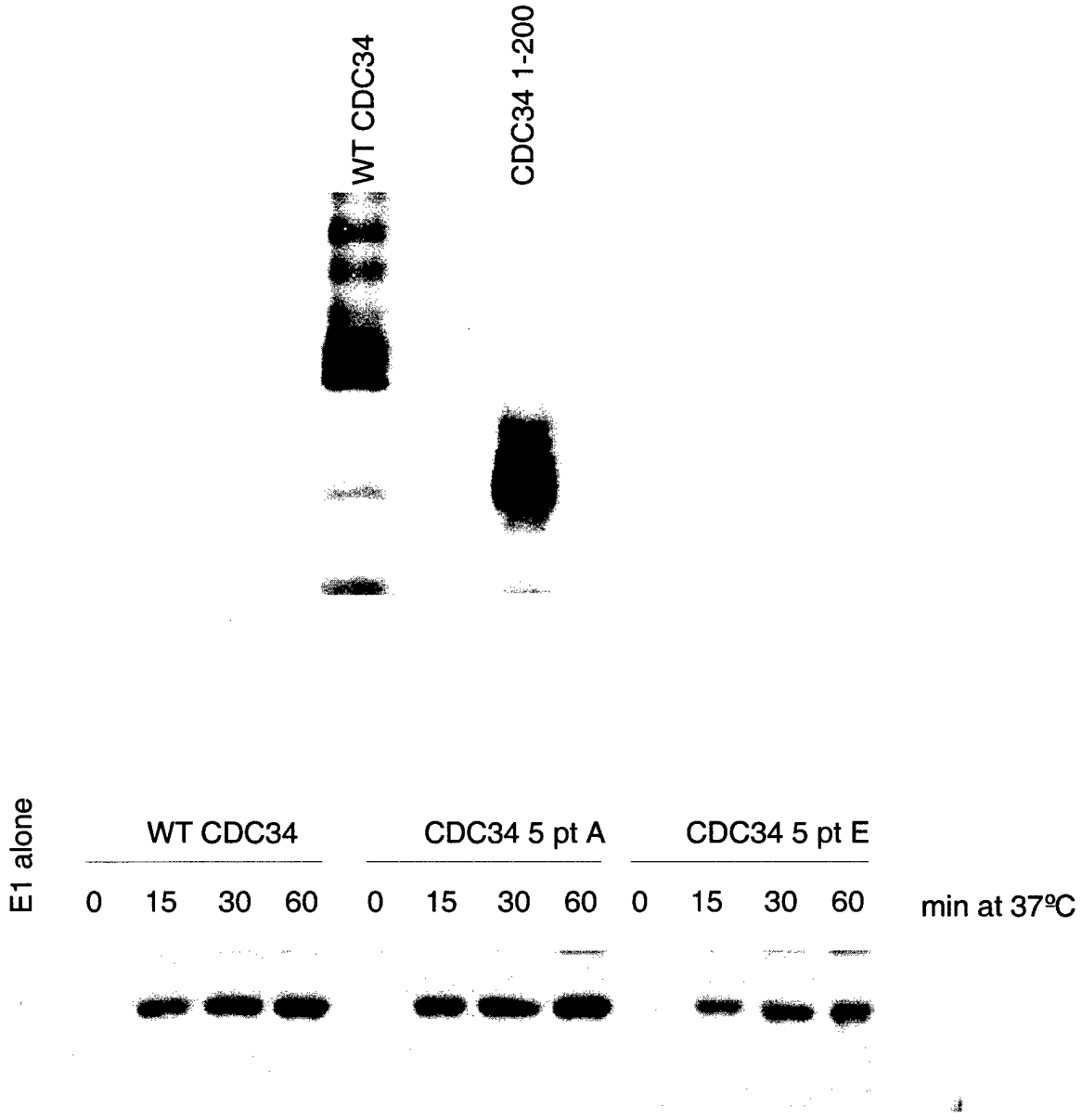
Immunopurified CDC34 from breast cancer cell lines does not show an increased phosphorylation. (IP-Kinase-IP) CDC34 was immunoprecipitated with affinity purified CDC34 antibody (CDC34 Ig) or non-immune rabbit antibody (RIg) followed by an in vitro kinase assay followed by another immunoprecipitation (IP-KINASE-IP) in normal breast cell lysate (lanes 1-4) or established breast cancer cell lysates (lanes 5-10)

FIGURE 3



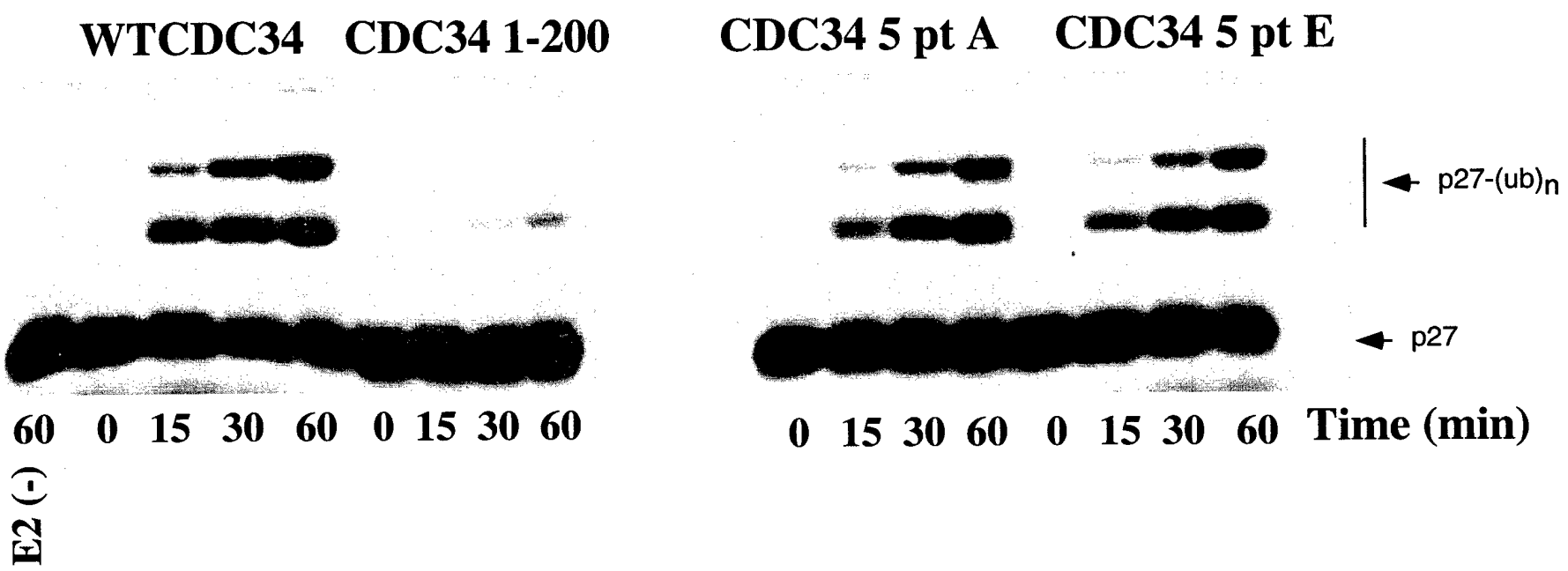
Amino Acids 200-236 of the tail domain of hCDC34 are required complementation of a cdc34-2 temperature sensitive strain in budding yeast.
Human CDC34 wildtype and mutants were cloned into pSF315b for complementation studies using *cdc34-2* temperature sensitive strain. pSF315b plasmids were transformed into yeast strains using lithium acetate and then were analyzed for growth at 25°C (Top left panel) and 37°C (bottom right panel) on leucine-minus minimal plates supplemented with galactose

FIGURE 4



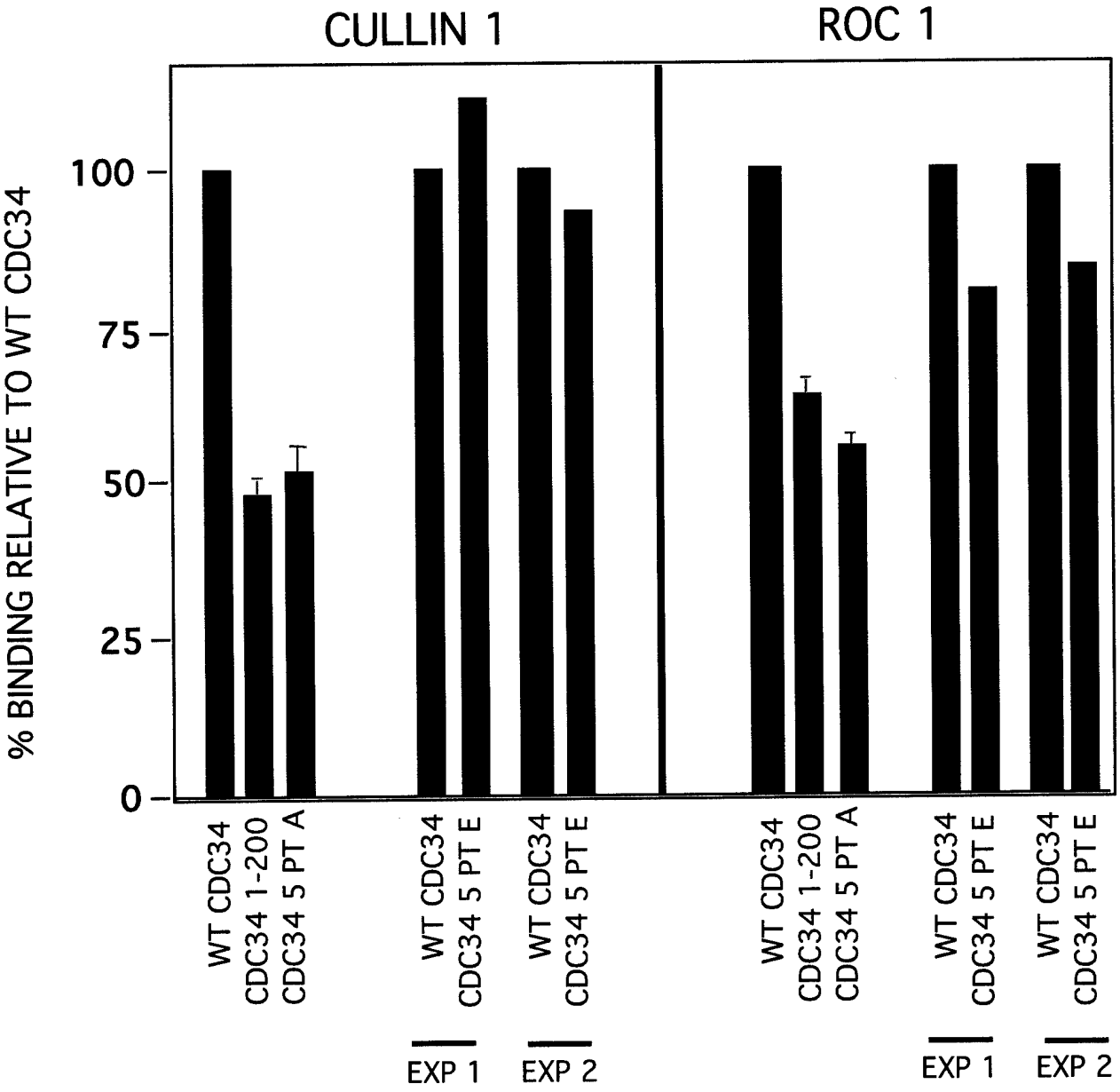
E2 Charging Assay. Purified 6xHis CDC34 WT and truncation CDC34 (1-200) were subjected to an E2 charging assay (top panel) or Purified 6xHis CDC34 WT and 6xHis CDC34 phosphomutants (bottom panel) and E2 charging assay performed at 37 C. Samples were removed at 0, 15, 30, 60 min.

FIGURE 5



The tail domain of hCDC34 is required for p27 ubiquitination. In vitro translated p27Kip1 was incubated in reaction mixture using WTCDC34, a truncation mutant of CDC34 (1-200), a phosphomutant of CDC34 (5 pt A) or a glutamic acid mutant (5 pt E) as the ubiquitin conjugating enzyme. Samples were removed at 0. 15, 30, 60 minutes (min).

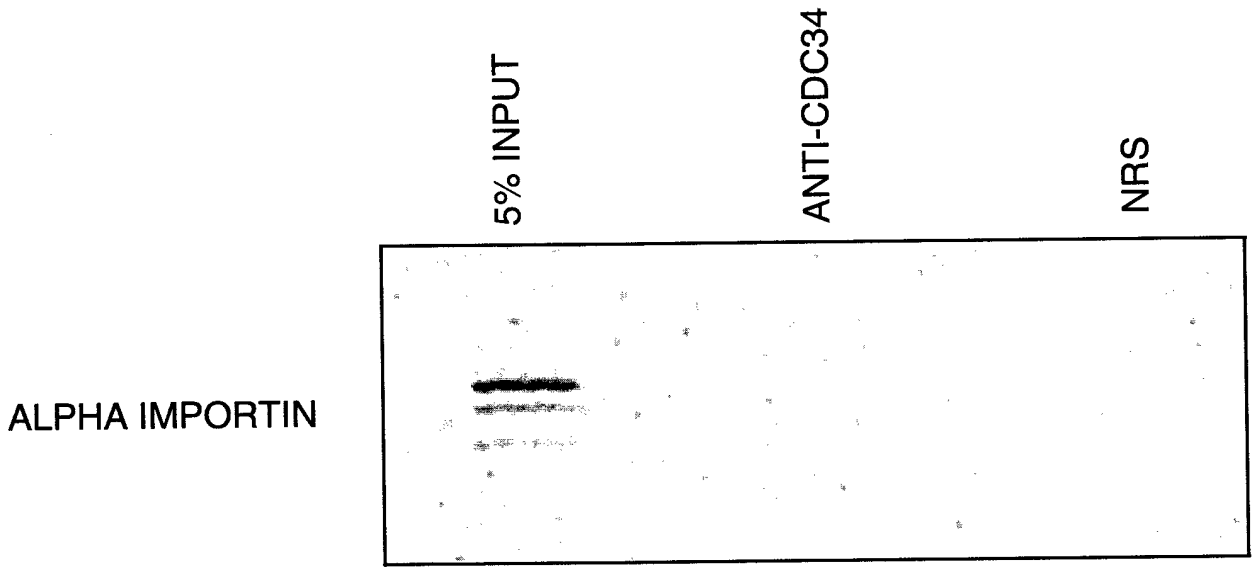
FIGURE 6



Co-immunoprecipitation assay. CUL1/ROC1 were co- in vitro translated with [^{35}S] methionine and incubated with purified 6xHis CDC34 WT, a truncation mutant (CDC34 1-200), a phosphomutant of CDC34 (5 pt A) or a glutamic acid mutant. The samples were then immunoprecipitated with CDC34 antibody (ANTI-CDC34) or normal rabbit serum (NRS). % binding of CDC34 mutants were calculated relative to WT CDC34 and the standard error of the mean was calculated shown by error bar. CDC34 5 PT E % binding was calculated relative to WT CDC34 in two independent experiments.

FIGURE 7

CO-IMMUNOPRECIPITATION



Human CDC34 does not interact with alpha importin in vitro. Co-immunoprecipitation assay.Alpha importin was in vitro translated with [^{35}S] methionine and incubated with purified 6xHis CDC34 WT. The samples were then immunoprecipitated with CDC34 antibody (ANTI-CDC34) or normal rabbit serum (NRS). 5% of invitro translated alpha importin is shown (5% INPUT).