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Disseminated malignancies are commonly treated with cytotoxic agents (e.g., chemotherapy, radiation) that target the unregulated growth associated with tumors. However, many of these procedures have proven unsuccessful due in part to the acquired resistance of cancer cells to these regimens. Mounting evidence suggests that one underlying mechanism by which malignancies are protected from cytotoxic agents is through aberrant activation of a pathway generally referred to as the "stress response". Using a genetic approach in yeast, we have identified a new C-type cyclin (UME3) that, when deleted, allows the inappropriate expression of the HSP70 family member SSA1. Several pieces of data suggest that the human cyclin C (cycC), which exhibits nearly 40% identity to the yeast gene, may also be involved in regulating the stress response. First cycC co-localizes with the human RNA polymerase suggesting a role for this cyclin in transcriptional regulation. Second, when expressed in yeast, cycC is rapidly destroyed in cultures exposed to elevated temperatures. Finally, we have mapped cycC to a region of the genome (6q21) that is frequently deleted in breast tumors. This proposal will explore the relationship between cycC activity, the stress response and drug sensitivity.

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Randy Stuck
Principal Investigator's Signature

8/25/99
Date

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(5) Introduction.

Disseminated malignancies are commonly treated with cytotoxic agents (e. g., chemotherapy, radiation) which target the unregulated growth associated with tumors. However, many of these procedures have proven unsuccessful due in part to the acquired resistance of cancer cells to these regimens. Mounting evidence suggests that one underlying mechanism by which malignancies are protected from cytotoxic agents is through aberrant activation of a pathway generally referred to as the "stress response". This system, which is found in all organisms from procaryotes to man, elicits the expression of several conserved gene families (heat shock proteins e. g., Hsp70, Hsp27) that protect the cell from cytotoxic agents. In human breast cancer, overexpression of Hsp's has been associated with tumors that are both more invasive and/or resistant to chemotheraputic drugs. We propose to expand studies initiated in the budding yeast *S. cerevisiae* to investigate the role of the human cyclin C in regulating stress response genes in breast cancer tissues.

(6) Body

Object 1: Is cycC down regulated as part of the normal cellular response to stress?

Task 1: Establish human cell lines stably expressing cycC.

Stable cycC transfectants were established in two separate cell lines, Hela and MCF7 breast cancer cells. The Hela cell line was chosen to distinguish between general effects on cycC regulation from those specific for breast cancer cells. First, the cycC cDNA was obtained from Dr. S. Reed, Scripts Institute (1). The cDNA was tagged with the HA epitope (2) using standard techniques. The tagged version of cycC was inserted into the vector pCDNA3 to form pKC414 (Fig. 1) under the control of the CMV promoter which provides high, constitutive expression in many cell types.

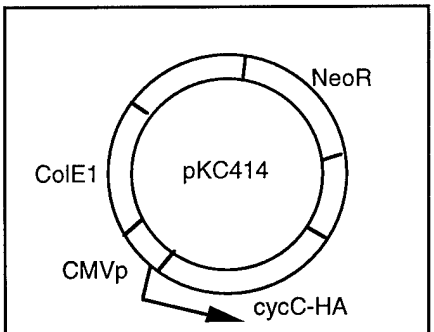

Figure 1. Map of pKC414

The pKC414 plasmid was stably integrated into both Hela and MCF7 cell lines by lipofection. Transfectants were selected for neomycin resistance and subclones isolated. The clones (minimum of 10) were pooled and expanded for the experiments described below. To determine the expression levels of cycC, Western blots were performed on the cell lines using standard protein extract preparations. A band corresponding to cycC was observed in the pKC414 transfected cell lines but not in the mock transfected control (Fig. 2). These results indicate that cycC-HA has been successfully integrated into these cell lines.

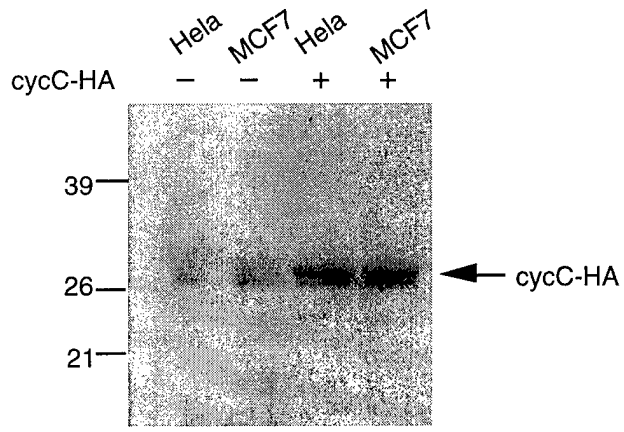

Figure 2. cycC-HA stable transfectants. Protein extracts were prepared from either pooled cycC-HA transfectants following neomycin selection (+) or the parental lines (–). Extracts were blotted and probed for cycC using HA monoclonal antibodies. cycC-HA specific bands are indicted by the arrow. Size standards (kDa) are presented on the left.

Task 2: Examine cycC regulation in response to stress agents, heat shock, nutrient deprivation and hypoxia.

Before cycC levels were determined in these cell lines in response to stress, the ability to evoke the stress response was determined. Antibodies directed toward the inducible form of Hsp70 were obtained from StressGen Inc. Both the Hela and MCF7 cell lines harboring the integrated cycC construct were expanded and split 48 hrs prior to testing to insure the cells were in exponential growth phase (data not shown). The cells were harvested and either protein extracts were prepared immediately, or and at various times following transfer to a 42°C water bath. Since the stress response is normally transient in nature, a timecourse was required to insure that the effects of excessive heat were not missed. Samples were taken prior to heat shock (0 hr) and at 2, 4, 6, and 8 hrs later. These times were successfully used in prior studies (3, 4). As expected, Hsp70 levels were low prior to heat shock (0 hr) but increased dramatically following exposure to elevated temperatures (Fig. 3). Due to a lack of starting material at the time of the experiment, the MCF7 sample did not have a 6 hr timepoint. Quantitation of these results indicated that Hsp70 was induced approximately 70-fold in these experiments (data not shown). Interestingly, unlike the Hela cells, the Hsp70 levels did not return to pre-heat shock levels in the MCF7 culture. Currently, it is not clear whether this represents a failure of this cell line to correctly respond to heat shock or normal experimental variation.

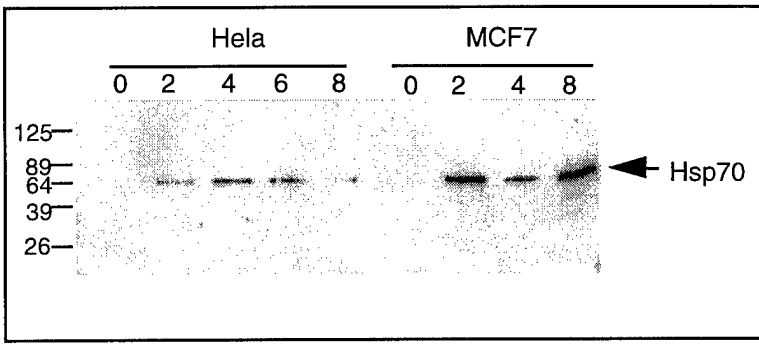

Figure 3. Induction of Hsp70 in response to heat shock. Cell lines indicated containing the cycC-HA construct were subjected to heat shock (42°C) for the times indicated (hrs). Extracts were prepared and 0.5 µg of protein were blotted and probed for Hsp70. Size standards are given on the left (kDa).

Our working model predicts that cycC represses Hsp70 expression. Moreover, this repression is relieved by destroying the cyclin in response to stress. Therefore, we examined cycC levels during the early timepoints of Hsp70 induction. Western blots were performed with extracts representing 0, 1 and 2 hrs following exposure of cells to elevated temperatures. These studies indicated that cycC levels were not significantly affected in either Hela or MCF7 cells (Fig. 4). Similarly, no difference in cycC levels were observed in cells exposed to stress inducing levels of ethanol or hydrogen peroxide (data not shown). These results suggest that unlike the yeast C-type cyclin, human cycC levels are not affected by stress or that the conditions used in this study were not correct to detect any changes in cycC levels.

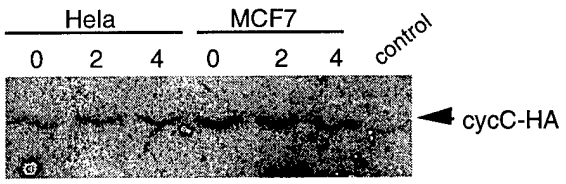

Figure 4. cycC levels in response to heat shock. The cell lines indicated containing cycC-HA were subjected to heat shock (42°C) for the times indicated (hrs). Protein extracts were prepared and subjected to Western blot analysis with the HA monoclonal antibody. Control sample contains extract prepared from the parental MCF7 cell line lacking cycC-HA. cycC-HA specific signal is indicated by the arrow.

One significant difference in the experimental protocols used to examine the stress response in yeast and mammalian cells is the use of a recovery time following treatment in the latter system. The rationale behind recovery time is not clear but involves returning the cells to pre-stress conditions and is standardly used as the induction of many stress response genes does not occur until this period. Therefore, cycC levels were examined under conditions that used a recovery period with media containing FBS. The results of these experiments are depicted in Fig. 5. MCF7 cells were harvested, split and protein extracts were prepared immediately for the pre-heat shock control containing one half of the culture (0 hr). The remainder of the cells were subjected to heat shock (42°C) for 2 hr then allowed to recover in medium + FBS for 4.7 hr. Western blots probing for cycC of two dilutions of the extracts revealed that cycC levels were significantly reduced compared to the untreated control. These results indicate that cycC is down regulated in response to heat shock but only during the recovery period.

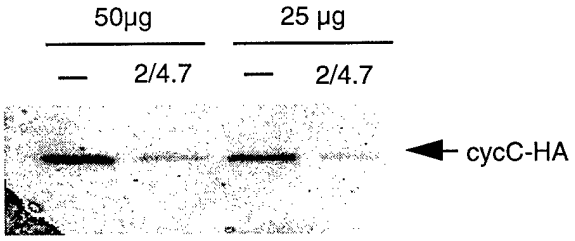

Figure 5. cycC levels following heat shock recovery. MCF7 stably transfected with cycC-HA were untreated (–) or heat shocked for 2 hrs followed by 4.7 hr recovery at 37°C. Extracts were prepared and the indicated amount of protein was subjected to Western blot analysis.

Task 3: Examine cycC regulation in response to cytotoxic agents doxorubicin, taxol, and 5-fluoro-uracil.

Due to the wide range of response kinetics observed for different stressors, an alternative approach was utilized to rapidly determine if cycC levels were affected by stress. This approach utilized tumor necrosis factor alpha or TNFα. TNFα stimulates the Jun activated kinase (JNK) cascade which triggers a general stress response. To examine whether TNFα treatment affected cycC levels, the following experiments were performed. MCF7 cells transfected with cycC-HA were treated with TNFα (2 μg/ml) for 48 hrs. Control flasks were maintained for the same period but TNFα was omitted. The results of these experiments suggest that cycC is down regulated following exposure to TNFα compared to the untreated control. These findings support our previous conclusions that cycC levels are affected by the cellular stress response pathways.

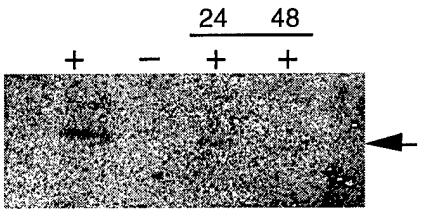

Figure 6. Regulation of cycC by TNFα. MCF7 cell line harboring cycC-HA (+) were subjected to TNFα treatment for the times indicated (hrs). The mock transfected parental line (–) serves as a control for non-specific binding of the HA Mab.

Task 4. Using directed and random mutagenesis, the cis-acting sequences the mediate cycC destruction will be sought.

The results from several experiments indicate that the stabilization of Ume3p derivaitves mutated in the CBE is not due to a defect in Cdk binding. Since stress represents a new regulatory signal governing the stability of cyclins, it may not be too surprising that a novel destruction element is involved. In addition, our finding that the A110V mutation is also able to protect Ume3p from meiosis-induced destruction [Cooper, 1997 #851] places the CBE at the intersection of a previously unknown merging of the regulatory systems governing meiosis and the stress response. Once the CBE is completely defined in yeast, the corresponding residues will be altered in cycC. The effects of these mutations on cycC destruction in response to heat shock and TNFa treatement will be deteremined.

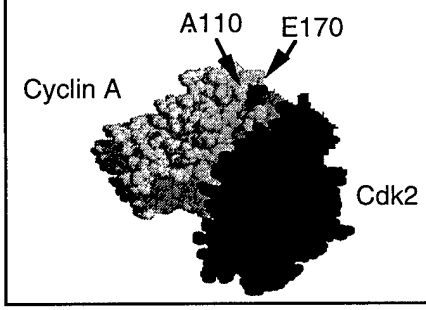

Figure 6. Cyclin Box Element (CBE). The location of the A110 and E170 residues (arrows) as modeled on Cyclin A coordinates (see text for details). The position of Cdk2 is also indicated. The structure shown is based on cyclin A residues 173-432; Cdk2 residues 1-298 (full lenght). Modeling Ume3p sequences on the more closely related human cyclin H produced similar results.

Objective 2: Does cycC activity effect drug sensitivity in transformed or non-transformed breast cell lines?

Task 5: Examine effect of overexpression of cycC on drug resistance.

Two vectors were constructed that overexpressed cycC in cos cells. These plasmids contained an SV40 origin for plasmid replication and expressed cycC from the CMV promoter described above. The transient transfectants (and vector alone controls) were treated with three drugs used in breast cancer regimens namely doxorubicin, taxol, and 5-fluoro-uracil. No difference in viability was observed in pooled transfected exposed to either of these drugs compared to the mock transfected control (data not shown). These results indicate that overexpression of cycC alone is insufficient to cause a significant change in drug sensitivity. However, since cycC activates the cyclin dependent kinase Cdk8 (8), overexpressing the cyclin alone may not be functionally relevant. Therefore, experiments are ongoing in which the cycC and Cdk8 will be simultaneously overexpressed and the drug sensitivities re-examined.

Task 6. Examine the role of cycC in HSP gene expression.

No experiments have been completed toward this task.

Task 7: Examine drug resistance of tumor cells deleted for 6q21 with or without cycC expression.

No experiments have been completed toward this task.

(7) KEYRESEARCH ACCOMPLISHMENTS:

- CycC levels are reduced in response to heat shock and TNFα treatment
- A new destruction element (CBE) has been identified that is required for the destruction of the yeast C-type cyclin in response to stress and differentiation cues.

(8) Reportable outcomes.

Abstracts: Stress-Induced Destruction of C-Type Cyclins in Yeast and Man (D-14). 1996. Yeast Genetics & Human Disease. Baltimore, MD

Cell Lines. Two cell lines, Hela and MCF7, have been established that express a stably integrated cycC-HA tagged construct.

(9) CONCLUSIONS:

Similar to the findings in yeast, the human cycC levels are influenced by stress. This conclusion has been shown in two ways. First, cycC levels are reduced in cells exposed to elevated temperatures. Second, treating cells to TNFα, a known activator of

the stress response pathway, also reduces cycC concentrations. The next step will require the actual half life of cycC be determined using pulse chase experiments to ascertain whether the loss in cyclin levels is due to changes in stability or in translation efficiency.

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