

Award Number: DAMD17-01-1-0410

TITLE: Molecular Characteristics of Multicorn, a New Large
Proteolytic Assembly and Potential Anti-Cancer Drug Target,
in Human Breast Cancer Cells

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REPORT DATE: May 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20031106 037

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2003	3. REPORT TYPE AND DATES COVERED Annual (1 May 02 - 30 Apr 03)	
4. TITLE AND SUBTITLE Molecular Characteristics of Multicorn, a New Large Proteolytic Assembly and Potential Anti-Cancer Drug Target, in Human Breast Cancer Cells			5. FUNDING NUMBERS DAMD17-01-1-0410	
6. AUTHOR(S) Maria E. Gaczynska, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas Health Science Center at San Antonio San Antonio, Texas 78229-3900 E-Mail: gaczynska@uthscsa.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) <p>We have discovered a new giant proteolytic complex distinct from the proteasome and ubiquitous among Eukaryotes. The enzyme named multicorn apparently takes part in the cell cycle regulation and is involved in partial overcoming the physiological effects of proteasome inhibitors. As the most significant accomplishment, we wish to report here the development and successful testing of AAF-CMAC, an <i>in vivo</i> fluorescent probe of peptidolytic activity of multicorn. Its fluorescence arises from a CMAC group released from AAF, the peptide determining specificity of the substrate. We established conditions for its observation <i>in vivo</i> and found that the incubation with AAF-CMAC did not induce apoptosis. The overall intensity of fluorescence of CMAC in nonsynchronous MCF10A cells is 5 – 10 fold higher than in nonsynchronous MCF7 cells. The detected fluorescence followed the pattern of activity determined <i>in vitro</i> on the basis of subcellular fractionation. Additionally, we found the multicorn in a nuclear envelope. This localization is especially apparent in early mitotic cells and is clearly connected to the phosphorylation of the multicorn subunit. Since mitosis is the period of greatest vulnerability of cells to anti-cancer drugs, our observations strengthen the position of the multicorn as an attractive potential drug target alone or in combination with the proteasome.</p>				
14. SUBJECT TERMS Breast cancer			15. NUMBER OF PAGES 22	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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INTRODUCTION

Proper regulation of cell division and cell differentiation are the major factors preventing the neoplastic growth. Proteolysis is one of the widely accepted controlling mechanisms of these processes. Transcription factors and cell cycle regulatory proteins have to be activated or removed in a precise and timely fashion during the life of the cell. Largely controlled proteolysis in cytosol and nucleus is executed by the giant multifunctional enzyme named the proteasome. The proteasome is an acknowledged anti-cancer drug target and one of specific inhibitors of the proteasome is now under extensive and successful clinical trials against multiple cancers, including breast cancer. We have discovered a new giant proteolytic complex distinct from the proteasome and ubiquitous among Eukaryotes (Osmulski and Gaczynska, 1998). The enzyme named multicorn apparently takes part in the cell cycle regulation and is involved in partial overcoming the physiological effects of proteasome inhibitors (Glass et al., 1998). Up to date, we found significant differences in the activity, amount, oligomerization status, posttranslational modifications and subcellular localization of the multicorn in human breast cancer MCF-7 cells, as compared with non-cancerous MCF-10A cells. We monitored the activity, composition and distribution of the multicorn on different stages of the cell cycle in the two cell lines. Apparently, the multicorn is also present in the nuclear fraction and associates with the subcellular membrane, most probably on the outside of the endoplasmic reticulum. Thanks to the unique tool, a fluorogenic multicorn substrate especially well suited for the *in vivo* studies and synthesized in our laboratory, we were able to directly observe the activity of the multicorn in living cells, follow the subcellular distribution of the enzyme, and detect the nuclearly localized multicorn mostly in nuclear envelope. Significantly, the nuclear envelope localization is especially apparent in early mitotic cells and is clearly connected to the phosphorylation of the multicorn subunit. Since mitosis is the period of greatest vulnerability of cells to anti-cancer drugs, the multicorn can be considered an attractive potential drug target alone or in combination with the proteasome. Our findings of sharp differences in the multicorn physiology between the cancerous and non-cancerous cell lines confirm that the multicorn can be also a useful marker of neoplastic transformation. We will continue our studies on the role of multicorn in cell cycle regulation and on mechanisms underlying the regulation of the multicorn activity.

BODY

Up to date, the research accomplishments associated with the objectives and tasks outlined in the approved Statement of Work are as follows:

Objective 1. Cloning and expressing the gene of human multicorn monomer.

Task 1: months 1 - 12; molecular basis of different physical and chemical properties of the two multicorn subunits will be studied using a combination of peptide mapping, sequencing and mass spectroscopy.

The task is near completion. We established that the human multicorn is built from a single subunit, similarly to the previously described by our group multicorn from fission yeast (*Schizosaccharomyces pombe*). The single subunit has apparent molecular mass 150

kDa. The 150 kDa subunit can be phosphorylated on several distinct serine residues to render polypeptides of electrophoretic mobility 165 kDa, 170 kDa and 240 kDa.

Currently, we purified the multicorn subunits to determine the sites of phosphorylation and the extent of the modification with mass spectroscopy. The pattern of phosphorylation is far from a simple "one modification site" model. This makes the multicorn control even more exciting, taking into account the regular, cell cycle - dependent pattern of changes in subcellular localization of differently modified multicorns we described below.

Task 2: months 1 - 6; cloning the gene, or genes, encoding the human multicorn monomers using HeLa cDNA library and PCR technology. It will be determined if the two types of subunits are encoded by the same gene.

As reported before, we established that the 150 kDa and 170 kDa subunits have to be encoded by the same gene, since the 170 kDa polypeptide is a posttranslationally modified form of the 150 kDa protein.

We cloned the gene coding multicorn in *S. pombe* and successfully induced its expression in a cell free system. On the basis of a set of biochemical tests, we concluded that there is a protein in mammalian cells matching nearly exactly properties of the yeast multicorn. Therefore, we initially planned to clone the mammalian homolog using the known nucleotide sequence of the yeast protein. Several attempts to obtain a full length or even truncated coding fragment of multicorn gene did not bring any meaningful results. As we cannot completely exclude a deeply embedded error in the design of our cloning approach, we are more tempted to believe that multicorns from these two sources share only a limited sequence homology not useful for the applied cloning strategy. The anticipated size of the gene (about 4 kbase) and the presence of a pleckstrin-like domain (PH) constitute additional difficulties.

Therefore, we plan following approaches to solve this problem:

1. Protein BLAST (NCBI) searches for the multicorn homologs in mammalian species did revealed recently two candidates with rather weak homology in its N-terminal domain (residues 1-400: about 30%) and a poor homology in its C-terminal domain (residues 800-950: less than 20%). The first is a KIAA1716 protein, accession BAB21807. This protein is of substantially shorter sequence than the expected (only 804 residues vs. 1300), but its start codon has not been identified yet. Its function has not been determined either. The second candidate is found in *Bos taurus*. It also contains the PH domain and its full length approximates well the expected size of the multicorn subunit (1129 residues), and has been isolated as DEF-1 protein promoting adipogenesis. These two proteins do share some amino acid sequence homology resulting mostly from the presence of PH domain and protein kinase binding domain.

2. A protein BLAST search against the *S. cerevisiae* database indicated yet another possible lead: a Sip3p protein interacting with SNF1 kinase (NP_014142). It consists of 1229 residues. A validity of this lead will be tested in next few weeks.

3. Recently, using the DNA BLAST search engine, we found a weakly homologous sequence of human DNA from clone RP11-87M1 on chromosome 9. As the homology is weak, it is evenly spread over the entire sequence of its *S. pombe* counterpart; therefore, it may constitute the key to our success.

4. We are also vigorously following the plan to obtain longer, and more useful amino acid sequences from the pure multicorn isolated from HeLa cells. Both, our Institute and Core Facility at UTHSCSA are equipped with the state-of-the-art instruments and staffed with the renowned specialists capable to obtain sequencing data of excellent quality from complex mixture of giant proteins.

We will try to use these new leads to finally accomplish our goal to clone the gene of multicorn.

Task 3: months 6 - 18; expressing the gene(s) in Schizosaccharomyces pombe or mammalian expression system. The goal of this task is to obtain active recombinant multicorn molecules characterized by controlled content of the specific monomers.

The work on this task will begin as soon as the cloned gene encoding the multicorn subunit will be available. The cause for the temporary delay in realization of this task is justified above (see Objective 1, Task 2).

Objective 2. Studying the mechanism controlling the multicorn activity through its oligomerization and phosphorylation.

Task 1: months 6 - 24; The multicorn complexes of different supramolecular organization and posttranslational modifications will be separated and purified. The qualitative and quantitative parameters of proteolysis catalyzed by these distinct complexes will be determined using selected protein substrates and model fluorogenic peptide substrates.

A significant part of this task has been already completed and described in previous Report. We found that the large form of the multicorn (about 4,000 kDa) is built mostly from the 170 kDa and small amounts of the 165 kDa and 240 kDa phosphorylated forms of the 150 kDa multicorn subunit. We found that the small form of the multicorn (900 kDa) is assembled mostly from the phosphorylated 240 kDa and non-phosphorylated 150 kDa polypeptides, and trace amounts of the phosphorylated 170 kDa protein. At this point of the studies, we are purifying the differently phosphorylated subunits of the multicorn to perform mass spectroscopy and identification of specific phosphorylation sites.

We found both the large (about 4,000 kDa) and the small (900 kDa) forms of the multicorn complex in MCF-7 and MCF-10A cells. We purified the two forms by a set of differential centrifugations combined with gel filtration and anion exchange chromatography (Gaczynska et al., 1993; Glas et al, 1998). Specifically, we enriched the cytosolic fractions of the cells with the large protein assemblies using 5 – hour ultracentrifugation at 100,000xg (at 4°C). The resulting protein pellet was resolubilized and subjected to gel filtration chromatography on a Superose 6 column (Pharmacia), especially designed for the separation of the large biomacromolecules with high resolution (Osmulski and Gaczynska, 1998). Further purification of the multicorn was carried out by anion exchange chromatography on the HQ/M column (Applied Biosystems). As we already established, the following fractions of the cells serve as a source of the specific phosphorylated subunits:

- Cytosol of MCF10A or MCF7 cells: the 240 kDa, 165 kDa and 170 kDa subunits;

- Membrane fraction: the 179 kDa, 165 kDa and 150 kDa subunits (the latter non phosphorylated, isolated from overconfluent MCF7 cells);
- Nuclear fraction of MCF10A: 170 kDa subunit.

As soon as the subunits will be purified in adequate supply, they will be separated in SDS-PAGE gels, in – gel digested by proteomix-grade trypsin and the digestion fragments will be subjected to mass spectroscopy.

Objective 3. Molecular characterization of the multicorn at different stages of the cell cycle. *Armed with our experience with the fission yeast multicorn, we will test how the activity, oligomerization status and posttranslational modifications of the multicorn change during cell cycle progression in human breast cancer MCF-7 cells and non-cancerous breast cells MCF-10A.*

Task 1: months 12 - 18; we will perform flow cytometric analysis of nonsynchronous MCF-7 and MCF-10A cells stained with anti-multicorn antibodies and with propidium iodide (DNA).

The work on this task started recently and is progressing in parallel to the studies on subcellular localization performed with the fluorogenic model substrate (see below).

Task 2: months 18 - 36; we will analyze the expression and biochemical properties of the multicorn in synchronized MCF-7 and MCF-10A cells.

A major part of the task has been already completed and was described in previous Report. We discovered that the subcellular localization of the multicorn depends on the cell cycle stage: the multicorn was detectable in the nuclear fraction only during mitosis, and only in the control, MCF-10A cells. We found that the pattern of differently phosphorylated subunits is specific for the particular cellular fraction, and differs between nonsynchronous, mitotic and overconfluent (G0) cells. During the period covered by this Report we supplemented the data obtained by Western blotting and proteolytic activity measurements in isolated subcellular fractions from MCF-10A and MCF7 cells with the data obtained *in vivo* with the fluorogenic model substrate. The substrate has been designed and synthesized in our laboratory especially for observation of the multicorn activity in living cells. The use of this unique tool already allowed us to discover that the nucleus-related localization of the multicorn is in fact restrained mostly to the nuclear envelope during the onset of mitosis.

Detection of multicorn peptidase activity in the MCF cell lines using model fluorogenic substrates.

To better characterize subcellular localization of multicorn and its changes during cell cycle progression in the MCF cell lines under *in vivo* conditions, we decided to use a model fluorogenic peptide as a probe detecting the peptidolytically active multicorn. For this purpose, we employed AAF-MCA, the best so far substrate for multicorn. However, the results were not impressive since an MCA group freed from AAF diffused easily from treated cells. Therefore, we turned our attention to coumarin derivatives, which are characterized by a better retention in living cells but still showing a high fluorescence yield and, after coupling to a peptide moiety, reasonable well soluble in water based solvents. Probably the most widely used derivative of

methylcoumarin, which meets the above criteria, is 7-amino-4-chloromethylcoumarin (CMAC). We successfully attempted to synthesize such a model fluorogenic peptide based on a well-known unblocked AAF moiety.

Synthesis of H-AAF-CMAC

Amino acids and peptide synthesis reagents were purchased from Advanced ChemTech or from Sigma/Aldrich. Solvents were obtained from Fisher and Fluka. CMAC was purchased from Molecular Probes.

All products were analyzed using reverse-phase high performance liquid chromatography (RP-HPLC), column: XTerra RP C₁₈, 5 μ m, 4.6 x 250 mm (Waters), developed with a gradient from 0 to 100% B, 30 min., where A: water + 0.1% trifluoroacetic acid (TFA), B: 80% acetonitrile/water + 0.1% TFA. A Delta 600E HPLC system from Waters was used to run and analyze chromatograms.

H-AAF-OH: Synthesis of H-AAF-OH was carried out on Advanced ChemTech Model 90 Peptide Synthesizer using Fmoc-strategy and preloaded Wang resin (Fmoc-Phe-Wang resin, 0.8 mmol/g).

Boc-AAF-OH: t-Butyloxycarbonyl (Boc) protecting group was coupled to the N-terminus of the peptide using the standard procedure described in the literature (Tarbell et al., 1971).

The solution of the peptide in a mixture of 1,4-dioxane, water and 1 M NaOH was stirred and cooled in an ice/water bath. Di-tert-butylpyrrocarbonate was added in portions, pH adjusted to 8-9, and stirring continued overnight at the room temperature. The solution was concentrated *in vacuo*, cooled in an ice/water bath, covered with a layer of ethyl acetate and acidified with 1 M potassium hydrogensulfate to pH 2-3. The aqueous phase was extracted with ethyl acetate. The organic extracts were pooled, washed with water, dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The residue was purified using RP-HPLC (XTerra Prep MS C₁₈ column, 5 μ m, 19 x 50 mm). The pure product (10.2 mg) was obtained. R_T = 25.69 min.

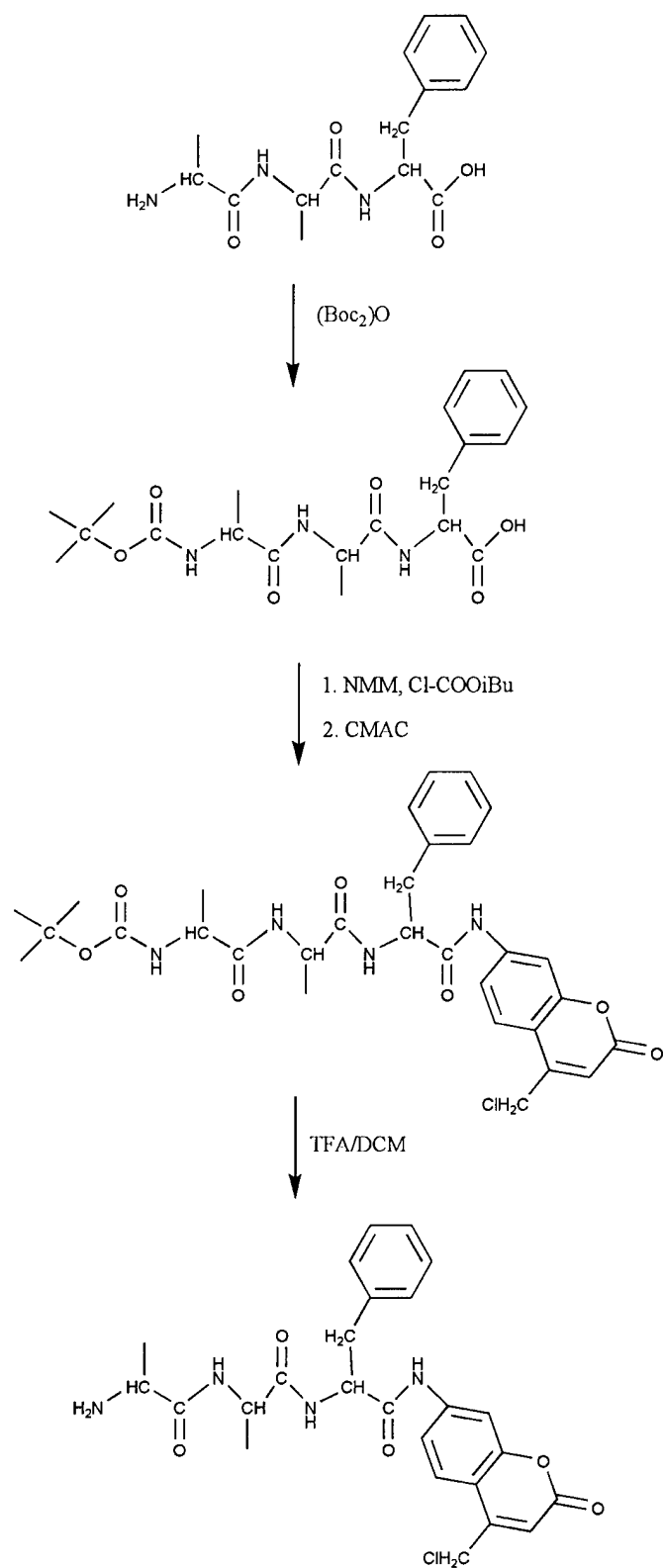
Boc-AAF-CMAC: Incorporation of CMAC moiety was done by the mixed anhydride procedure (Meienhofer J., 1979).

Boc-AAF-OH was dissolved in anhydrous tetrahydrofuran (THF) and cooled to -15°C (ice/ethanol). N-methylmorpholine was added followed by isobutyl chloroformate. The mixture was allowed to react in -15°C for 15 min. to form a mixed anhydride.

The solution of 7-amino-4-chloromethylcoumarin (CMAC) in anhydrous THF was added and the reaction mixture left stirring for 1 hour at -15°C and additional 2 hours at the room temperature.

The crude product was purified using RP-HPLC. 5.76 mg of the pure product was obtained. R_T = 29.16 min.

TFA x H-AAF-CMAC: Boc group was removed using 50% TFA/dichloromethane solution. The product was lyophilized giving 4.8 mg of foam. R_T = 22.11 min. MS: 499.15, calc. M = 498.96 Da.



Boc₂O - tert-butylpyrrocarbonate, NMM- N-methylmorpholine, Cl-COOiBu - isobutyl chloroformate, CMAC- 7-amino-4-chloromethylcoumarin, TFA - trifluoroacetic acid, DCM - dichloromethane

Figure 1

For biological studies, the probe was dissolved at concentration 10mM in DMSO (stock solution) and kept at -80°C . Directly before its use, AAF-CMAC was further diluted to 1mM with DMSO.

The probe shows absorption maximum at 354 nm and emission at 464 nm. A pilot study showed that AAF-CMAC is a good substrate recognized and digested by multicorn from *S. pombe*, HeLa, and MCF cells. An apparent fluorescent yield of CMAC is about three times lower than MCA giving the excellent readouts of the peptidase activity.

This unique tool was so far used to detect peptidase activity of multicorn in the nonsynchronous cells, cells arrested on the onset of mitosis with nocodazole and overconfluent cells representing G0 (quiescent) stage of the cell life. The experiments were performed as follows. MCF10A (control) and MCF7 (cancerous) cells were grown on coverslips to the desired stage (not confluent or overconfluent). To arrest the cells on the onset of mitosis, the nonconfluent cultures were treated with 0.15 micrograms/ml of nocodazole for 15 hours (Wang et al., 1997). For visualization of the multicorn activity, the coverslips were wet-mounted on microscope slides with 10 microliters of PBS (phosphate-buffered saline) containing 500 micromolar AAF-CMAC. Propidium iodide (PI) at the final concentration of 0.3 mg/ml was added to PBS to visualize dead cells easily permeable for PI. After 10 min. of preincubation at room temperature, the slides were analyzed under the fluorescent microscope (Zeiss AxioVision 2) with the excitation/emission filter used for DAPI visualization. The presence of propidium iodide bound to DNA was detected with the Texas Red filter of the microscope. Only less than 5% of the cells were permeable for the PI and this result confirmed that the incubation with AAF-CMAC did not cause apoptosis in the cells. To visualize DNA in the cells together with the multicorn activity, the coverslips with cells after at least 20 min incubation with AAF-CMAC were briefly washed with cold 50% ethanol, dried in air and wet-mounted on slides with PBS containing 0.3 mg/ml of PI. To specifically block the activity of the multicorn, cells on coverslips were first wet-mounted on slides with PBS containing 50 micromolar (final concentration) AAF-CMK (chloromethyl ketone derivative of the preferred-substrate AlaAlaPhe peptide), and after 10 minutes of preincubation at room temperature re-mounted on slides with AAF-CMAC or AAF-CMAC and PI, as above.

The following important observations emerged so far from the studies, which are vigorously pursued right now.

1. Degradation of the fluorogenic substrate AAF-CMAC by the multicorn was first assessed *in vitro* with purified samples of the protease. Then, the conditions were established to observe the activity *in vivo*, in MCF-10A and MCF-7 cells growing on coverslips and incubated with the substrate while wet-mounted on microscope slides. The concentration of the substrate and preincubation time used were sufficient for apparent saturation of the degradation reaction, since adding more of the AAF-CMAC or increasing preincubation time did not significantly change the observed intensity of the reaction. The fluorescence of fixed cells was stable for at least an hour of observation and no significant diffusion of the CMAC was observed.
2. Apparently, the incubation with AAF-CMAC did not induce apoptosis or otherwise harm the cells since we routinely observed no more than 5% of PI-permeable cells in the unfixed populations of MCF-10A and MCF-7 cells. Therefore, based on observation (1) and (2) we assume that the imaged distribution and intensity of fluorescence reflects the peptidolytic

activity of the multicorn in intact cells, presumably the phosphorylated large form of the protease, which displayed the highest activity in *in vitro* studies as described in the previous Report.

3. The images of MCF-10A and MCF-7 cells shown in Figures 2-5 have been brought to similar brightness by image processing software (Adobe Photoshop 5.5) for clarity and convenience of presentation. However, the overall intensity of fluorescence of CMAC in nonsynchronous MCF10A cells is 5 – 10 fold higher than the corresponding fluorescence in nonsynchronous MCF7 cells. This result is in perfect agreement with our data described in the previous report, where the activity of multicorn detected in isolated cytosolic fractions was up to 10 – fold higher in MCF10A, than in MCF7 cells. A more precise and detailed analysis of the intensity of fluorescence in the cells and cell compartments is in progress and will undoubtedly lead to further important observations.
4. The images of living cells degrading the fluorescent substrate of the multicorn clearly show the subcellular localization of the protease: cytosol, together with membrane structures, and nuclear envelope (Figure 2). The latter observation is an important progress from the previous Report when we relied on the results of fractionation of the cells into cytosolic, membrane and nuclear preparations, and distinguishing between the nucleoplasm and the nuclear envelope was impossible.
5. Consistently with our previous observations and predictions, we detected changes in subcellular localization of the multicorn activity during progression of the cell cycle when imaging nonsynchronous cells, cells arrested on the onset of mitosis with nocodazole, cells released from the nocodazole arrest, and overconfluent cells. Importantly, we were able to precisely follow the changes in both types of cells, especially the emergence and dispersion of the activity localized in and around a nuclear envelope.
6. Specifically, we established that (see Figures 2-5):
 - the activity was always present in the cytosol, albeit it was faint in overconfluent (G0) cells;
 - foci of strong CMAC fluorescence were detectable in the nucleus when the nuclear membrane was still present, but not in overconfluent (G0) cells;
 - a very strong fluorescence around/in the nuclear envelope appeared in late G2 and/or on the onset of mitosis and persisted until the nuclear membrane disappeared;
 - for a brief period during chromatin condensation a very strong fluorescence co-localized with the emerging chromosomes;
 - subsequently, on late stages of the condensation of chromatin, the fluorescence diffused into the cytosol leaving only a faint border around condensed chromosomes.

We noted the following differences between the intensity and distribution of CMAC fluorescence in MCF-10A and MCF-7 cells:

- the fluorescence around nuclear envelope appeared stronger in relation to the cytoplasmic background in nonsynchronous MCF-7 than in MCF-10A cells. The precise quantification of the fluorescence signal is in progress;

- the strong fluorescence around nuclear envelope seemed to appear already in G2 in MCF-7 cells, whereas it correlated rather with the onset of mitosis in MCF-10A cells. There were many more cells with well-visible fluorescence around nuclear envelope in nonsynchronous population of MCF-7 cells, than in MCF-10A cells (Figure 2). This important observation will be confirmed after an additional set of experiments with a flow cytometry detected distribution of multicorn in the cells stained with PI, AAF-CMAC, and fluorescent anti-multicorn antibodies.

7. As predicted, when the nonsynchronous cells were briefly treated with the multicorn inhibitor, AAF-CMK, before incubation with the fluorescent substrate, the overall intensity of the fluorescence was up to 100-fold lower than in control, non-inhibited cells. However, there were striking differences in distribution of fluorescence signal in MCF-10A and MCF-7 cells (Figure 5). Most striking, the fluorescence around nuclear envelope disappeared completely in the inhibitor-treated MCF-10A cells (non-cancerous), and only traces of the fluorescence in the cytosol remained. To the contrary, the inhibitor-treated cancerous MCF-7 cells retained their subcellular distribution of the CMAC fluorescence, only with the overall 40-60-fold decrease in intensity (Figure 5). The possible reasons for the observed phenomenon can be grouped as follows:
 - AAF-CMK can be overall less efficient in MCF-7 cells than in MCF-10A due to differences in cell membrane permeability or secondary reactions inside the cell inactivating the inhibitor before it reaches the nuclear envelope;
 - There can be an AAF-CMAC-degrading enzyme other than the multicorn especially active in MCF-7 cells, which can compensate for the inhibited multicorn activity. The latter possibility is especially intriguing and can be very important when considering the multicorn as a potential anti-cancer drug target.

Our model of localization and modifications of the multicorn in MCF-7 and MCF-10A cells. was developed on the basis of the data collected *in vitro*. Currently, we confirmed the proposed previously model *in vivo*, supplementing it with the observations that the multicorn accumulates in the nuclear envelope during onset of mitosis.

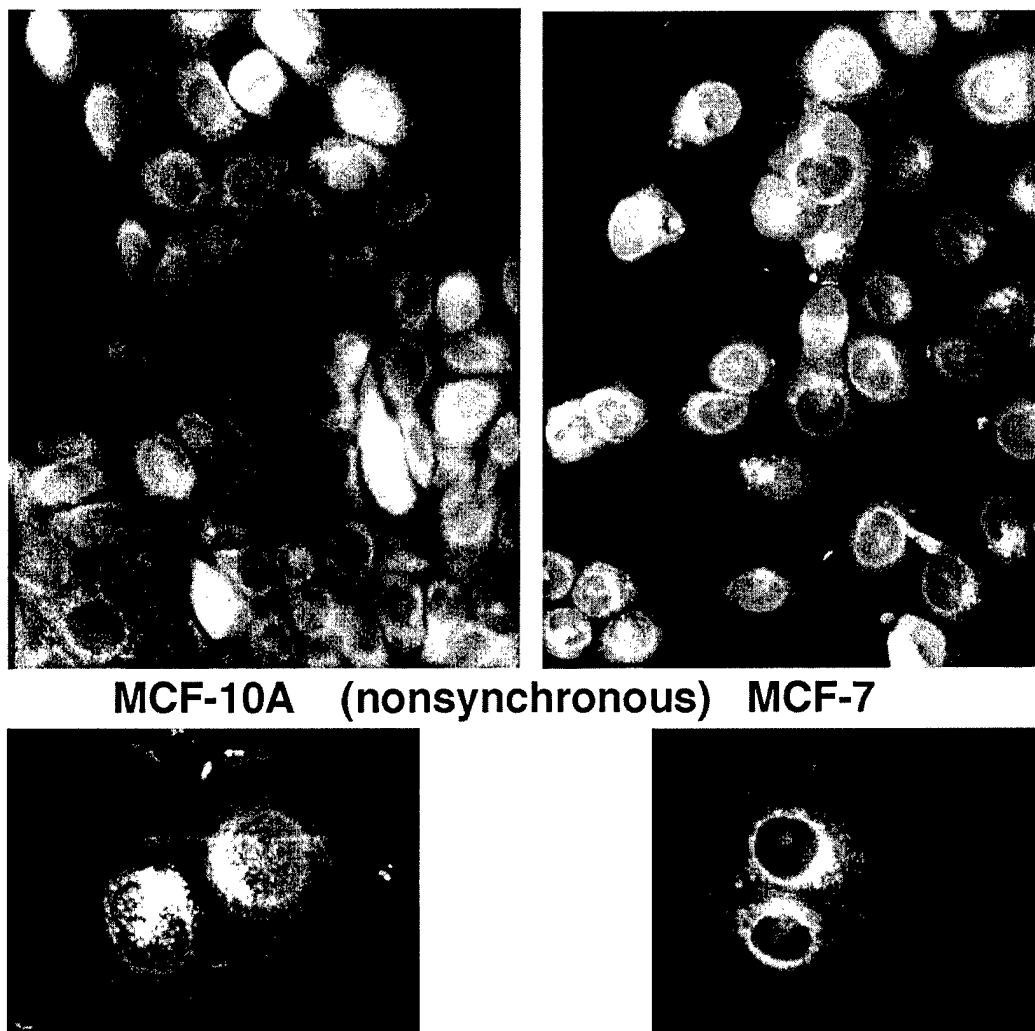


Figure 2. Nonsynchronous MCF-10A (control) and MCF-7 (cancerous) breast cells were incubated with fluorescent substrate of the multicorn AAF-CMAC. Top: fields of cells growing on coverslips (collected at magnification 400 x); bottom, close-up of cells just after cell division (collected at magnification 630x). The fluorescence of a free CMAC released from the substrate by peptidolytic activity of the multicorn was imaged under fluorescence microscope with the use of a blue filter. The fluorescence is visualized as white and shades of gray on a black non-fluorizing background. The relative intensity of gray shading in both images was artificially brought to similar brightness and contrast for the convenience of direct comparison in the Figure. However, the actual intensity of fluorescence of MCF-7 cells was several times lower than the intensity of fluorescence of MCF-10A cells. Nevertheless, the relative fluorescence around the nuclear envelope, as compared with the fluorescence of the cytosol, seems to be more prominent in MCF-7 than in MCF-10A cells.

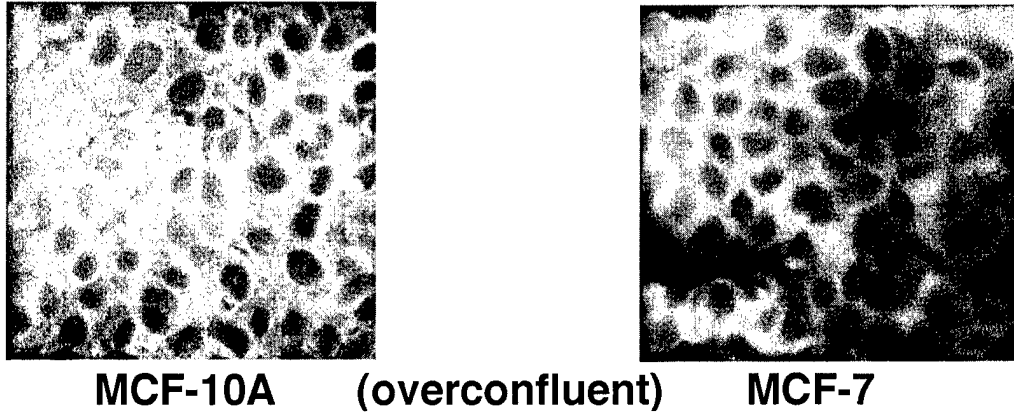


Figure 3. Overconfluent (G0) MCF-10A (control) and MCF-7 (cancerous) breast cells were incubated with fluorescent substrate of the multicorn AAF-CMAC. Only a relatively faint, dispersed fluorescence of free CMAC is visible in the cytosol of both types of cells. The dark ovals in the center of cells are nuclei, as was assessed with nucleic-acid staining fluorescent dye propidium iodide. The images were collected with 400x magnification.

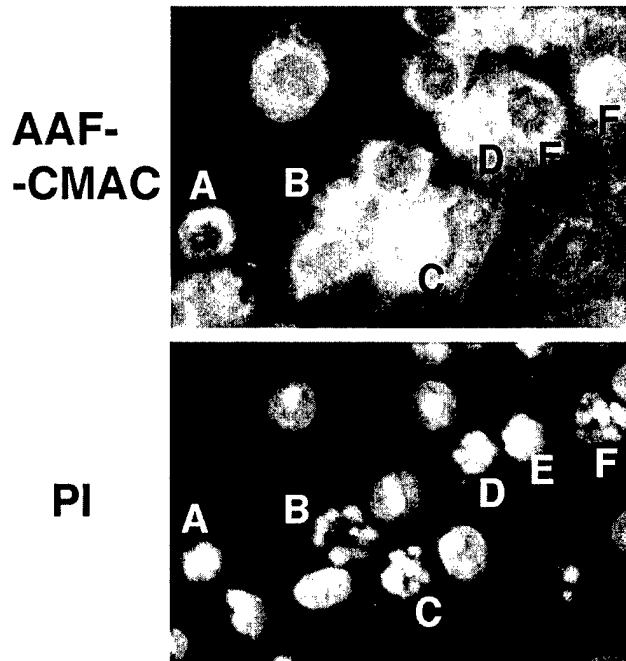


Figure 4. The activity of multicorn changes its subcellular localization depending on the stage of cell cycle. A gallery of MCF-10A cells on different stages of mitosis is presented (images collected with 630x magnification). Top: cells treated with AAF-CMAC and fixed, bottom, the same cells stained with PI to visualize DNA. Examples of cells: A, E - chromatine condensed, CMAC fluorescence disppersed in cytosol and faintly bordering chromosomes; B, C, D, F - chromatine on early stages of condensation, CMAC fluorescence very strong and surrounding the chromatin.

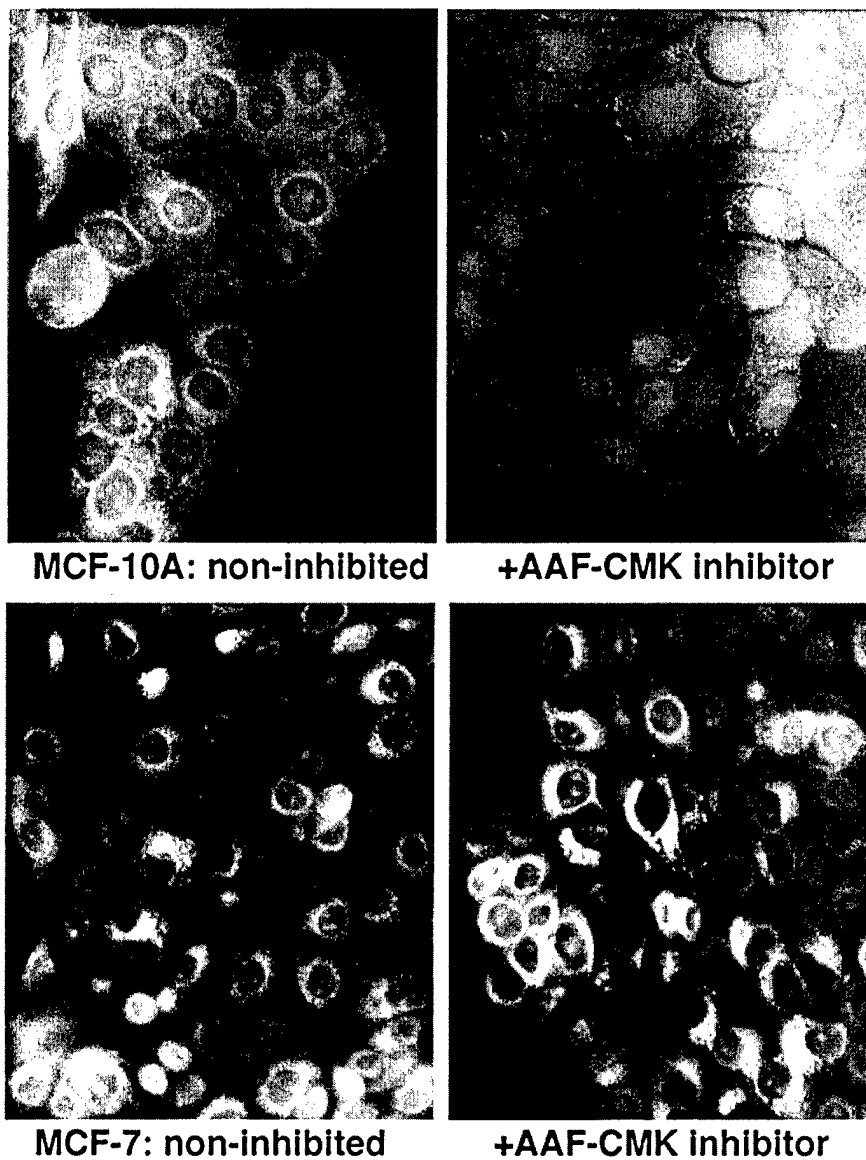


Figure 5. MCF-10A and MCF-7 cells respond differently to the treatment with the multicorn inhibitor AAF-CMK. The relative intensity of gray shading in both images was artificially brought to similar brightness and contrast for the convenience of direct comparison in the Figure. The actual intensity of fluorescence of non-inhibited cells was up to two orders of magnitude higher than that of the inhibitor-treated cells.

KEY RESEARCH ACCOMPLISHMENTS

(the accomplishments achieved specifically during the recent year are **in bold**)

- We established that the human multicorn is built from a single subunit of apparent molecular mass 150 kDa. We found that the 150 kDa subunit can be phosphorylated on serine residues in several distinct sites to render polypeptides of electrophoretic mobility 165 kDa, 170 kDa and 240 kDa.
- We discovered that the multicorn, which was first found and described in the cytosol, is present in the nucleus of mitotic cells. Importantly, the multicorn is detectable only in nucleus of the control MCF-10A cells, and not in the MCF-7 cancerous cells.
- We established that the pattern of differently phosphorylated subunits is specific for the particular cellular fraction, and differs between nonsynchronous, mitotic and overconfluent (G0) cells. The pattern in specific subcellular compartments differs between control and cancerous cells.
- We isolated the large and small oligomeric forms of the multicorn and determined their subunit composition in respect to differently phosphorylated polypeptides.
- We found that the large oligomeric form of the multicorn isolated from MCF-10A control cells exhibits several - fold higher specific activity toward a model peptide substrate than the large form isolated from breast cancer cells MCF-7. This difference is accompanied by a distinct pattern of subunit phosphorylation.
- We found that the ratio of large and small forms of the multicorn dramatically differ in cytosols of nonconfluent and overconfluent control MCF-10A cells. The large form was predominant in nonconfluent cells, whereas the small form was the only detectable active form of the multicorn in the cytosol of overconfluent (G0) MCF-10A cells. To the contrary, in cancerous MCF-7 cells the large cytosolic form was always predominant with only a small decline in overconfluent cell culture.
- **We synthesized and characterized a unique tool for studies of the multicorn in living cells, a fluorescence probe AAF-CMAC.**
- **The probe readily diffused into MCF cells, where was digested with the subsequent release of CMAC. The process did not induce apoptosis.**
- **The imaged distribution and intensity of fluorescence reflected the peptidolytic activity of the multicorn in intact cells, presumably the phosphorylated large form of the protease, which displayed the highest activity in *in vitro* studies.**
- **The overall intensity of fluorescence of CMAC in nonsynchronous MCF10A cells was 5 – 10 fold higher than in nonsynchronous MCF7 cells, what agrees perfectly with the *in vitro* measurements.**

- The multicorn was unevenly localized in the MCF cells: mostly in cytosol together with membrane structures, but also nuclear envelope.
- We detected changes in subcellular localization of the multicorn activity during progression of the cell cycle, which followed our previous observations of the multicorn distribution in *S. pombe*. The activity was always present in the cytosol, but only at a very low level in the overconfluent (G0) cells; Foci of strong CMAC fluorescence were detectable in the nucleus when the nuclear membrane was still present, but not in overconfluent (G0) cells; Moreover, a very strong fluorescence around/in the nuclear envelope appeared in late G2 and/or on the onset of mitosis and persisted until the nuclear membrane disappeared. For a brief period during chromatin condensation, a very strong fluorescence co-localized with the emerging chromosomes. Finally, on late stages of the condensation of chromatin, the fluorescence diffused into the cytosol leaving only a faint border around condensed chromosomes.
- The observed activity was inhibited *in vivo* by an exposure of the cells to AAF-CMAC, the only known inhibitor of the multicorn.

REPORTABLE OUTCOME

1. The research conducted on the project was a topic of the poster and a plenary talk on the Era of Hope DOD Breast Cancer Research Program Meeting (Orlando, September 25-28, 2002). The abstract is attached in APPENDICES.
2. The research was a topic of the poster **“Proteolytic instability in breast cancer cells.”** Presented on 2nd International Symposium on Cancer Research “Frontiers in Cancer Research: a Molecular Perspective” (San Antonio, October 12-14, 2002). The abstract for the poster by Pawel A. Osmulski, Xianzhi Jang, Bingnan Gu and Maria E Gaczynska is attached in APPENDICES.
3. The research was a topic of the poster on 12th Annual Symposium on Cancer Research in San Antonio (July 12th, 2002). The abstract for the poster: **“Cancer, proteases and proteolytic instability.”** by Pawel A. Osmulski, Xianzhi Jang, Bingnan Gu and Maria E Gaczynska is attached in APPENDICES.
4. Parts of the research were included in the plenary lecture **“The target and the bullets: aiming at the proteasome in cancer therapies.”** given at the 13th Annual Symposium on Cancer Research in San Antonio (July 12th, 2002).
5. Parts of the research were included in the invited lecture **“Carpet bombing or sniper fire: the two approaches in proteasome-targeting drug development.”** given at the Institute of Drug Development (San Antonio, January 22, 2003).

6. Postdoctoral Associate Elzbieta Jankowska is trained in biochemical and cell biology techniques as a part of conducted research. Dr. Jankowska, a chemist, synthesized the AAF-CMAC substrate, which proved to be an extremely useful tool for multicorn studies.

7. Graduate student in Molecular Medicine, Xiaolin Qin, is trained in a variety of biochemical and cell biology techniques in PI's laboratory.

CONCLUSIONS

We showed that breast cancer MCF-7 cells possess a distinct regulation of proteolysis executed by the multicorn when compared with non-cancerous MCF-10A cells. The apparent lack of the multicorn in the nucleus of MCF-7 cells may constitute an important link between the overall efficiency of cell division and nuclear proteolysis. The cellular distribution of the multicorn, similarly to the proteasome, is not limited to cytosol, however the most of the both proteases resides in this compartment. Regulation of the assembly of the large and small forms of multicorn is accomplished through a complex phosphorylation pattern of their subunits. Moreover, it seems that the phosphorylation also controls subcellular localization of the multicorn and ultimately its fate.

We developed and successfully tested AAF-CMAC, an *in vivo* fluorescent probe of peptidolytic activity of multicorn. Its fluorescence arises from a CMAC group released from AAF, the peptide determining specificity of the substrate. We established conditions for its observation *in vivo*. We found that the incubation with AAF-CMAC did not induce apoptosis. The overall intensity of fluorescence of CMAC in nonsynchronous MCF10A cells is 5 – 10 fold higher than in nonsynchronous MCF7 cells. The detected fluorescence followed the pattern of activity determined *in vitro* on the basis of the subcellular fractionation. Additionally, we found the multicorn in a nuclear envelope. This localization is especially apparent in early mitotic cells and is clearly connected to the phosphorylation of the multicorn subunit. The AAF-CMAC activity *in vivo* is inhibited by AAF-CMK. Since mitosis is the period of greatest vulnerability of cells to anti-cancer drugs, our observations strengthen the position of the multicorn as an attractive potential drug target alone or in combination with the proteasome.

On the basis of our data we suspect that multicorn constitutes an important player in cellular protein turnover probably also involved in regulation of cell cycle. Its distinct properties in the control and cancerous cells suggest that the multicorn may represent an attractive drug target and a marker of physiological state of the cells.

Future studies should indicate whether the multicorn participate directly in cell cycle regulation. The results of studies on the fission yeast homologue of the protease strongly support that possibility.

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APPENDICES

1. Abstract of the poster presented in the Era of Hope DOD Breast Cancer Research Program Meeting (Orlando, September 25-28, 2002).

PROTEOLYTIC INSTABILITY IN BREAST CANCER CELLS

**Maria E Gaczynska, Xianzhi Jang, Bingnan Gu,
Pawel A. Osmulski**

Protein degradation, in concert with protein synthesis, governs the proper execution of metabolic processes in the cell. The large, intracellular proteases like anti-cancer drug target proteasome, a novel protease multicorn, tripeptidyl peptidase II (TPPII) or leucine aminopeptidase (LAP) play a key but poorly understood role among all proteolytic enzymes due to their diverse functions. The proteasome actions are essential for cell cycle regulation, turnover of transcription factors and antigen processing. Inhibition of proteasome leads to cell death and is utilized to kill tumor cells. However, there are strong indications that the other proteases may also constitute valuable anti-cancer drug targets. The postulated duties of multicorn include degradation of cell cycle related factors and, together with TPPII and LAP, further processing of antigenic peptides produced by proteasome. The collaboration of proteasome and other large proteases suggests the existence of a net of functional relationships between the executors of controlled proteolysis.

We show here that proteolytic instability, which manifests in changing the equilibrium between the activities of large cytosolic proteases is one of the signs of neoplastic transformation in human breast cancer MCF7 cells, as compared with non-cancerous MCF10A cells. To demonstrate the proteolytic instability we quantified the amount of enzymes with Western blotting, and determined their proteolytic activities with high-throughput methods using specific fluorogenic peptide substrates.

In the functional proteomics fashion we found prominent changes on both the functional level of activities and specificities of the enzymes, and the structural level of subunit composition, modifications and subcellular distribution. Specifically, we found that there is markedly less proteasomes in nuclei of the cancerous than control cells, and nuclei of cancer and control cells have dramatically different pattern of subunits of a natural proteasome activator. Since proteasome, multicorn, TPPII and LAP all take part in antigen processing, the changes may impair removal of transformed cells by the organism. Probing the role of large proteases in maintaining the proper advance of cell cycle we discovered that the predominantly cytosolic multicorn can be found in the nucleus, but this residence is much more pronounced in cancerous than in control cells. Interestingly, both nuclei and cytosol of cancer cells contain mostly overphosphorylated, unstable forms of the protease. We postulate that the nuclear localization of multicorn holds the key for dissecting the role of this protease in cell cycle progression.

Our findings show a fragment of potentially critical interactions between parts of the controlled proteolysis machinery. This web of interactions should be taken into account when anti-cancer drugs target single components of the whole system.

The U.S. Army Medical Research Materiel Command under DAMD17-01-1-0410 supported this work.

2. Abstract of the poster presented in the 2nd International Symposium on Cancer Research "Frontiers in Cancer Research: a Molecular Perspective" (San Antonio, October 12-14, 2002).

PROTEOLYTIC INSTABILITY IN BREAST CANCER CELLS

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Protein degradation, in concert with protein synthesis, governs the proper execution of metabolic processes in the cell. The large, intracellular proteases like anti-cancer drug target proteasome, a novel protease multicorn, bleomycin hydrolase (BH), or leucine aminopeptidase (LAP) play a key but poorly understood role among all proteolytic enzymes due to their diverse functions. The proteasome actions are essential for cell cycle regulation, turnover of transcription factors and antigen processing. Inhibition of proteasome leads to cell death and is utilized to kill tumor cells. One of the synthetic proteasome inhibitors, a peptide boronate derivative PS-341 is already in clinical trials against multiple cancers, including breast cancer. However, there are strong indications that the other proteases may also constitute valuable anti-cancer drug targets. The postulated duties of multicorn include degradation of cell cycle related factors and, together with BH and LAP, further processing of antigenic peptides produced by proteasome. The collaboration of proteasome and other large proteases suggests the existence of a net of functional relationships between the executors of controlled proteolysis.

We show here that proteolytic instability, which manifests in changing the equilibrium between the activities of large cytosolic proteases is one of the signs of neoplastic transformation in human breast cancer MCF7 cells, as compared with non-cancerous MCF10A cells. To demonstrate the proteolytic instability we quantified the amount of enzymes with Western blotting, and determined their proteolytic activities with high-throughput methods using specific fluorogenic peptide substrates. In the functional proteomics fashion we found prominent changes on both the functional level of activities and specificities of the enzymes, and the structural level of subunit composition, modifications and subcellular distribution. Specifically, we found that immunoproteasomes, the specialized complexes especially well suited for antigen processing, are nearly absent in the cytosol of cancerous cells. Additionally, there is markedly less proteasomes in nuclei of the cancerous than control cells, and cancer and control cells have dramatically different pattern of subunits of a natural proteasome activator. We found that the new giant, predominantly cytosolic protease multicorn is found in the nucleus of cells arrested in pseudo-mitosis with nocodazole, however only in control and not in cancerous cells. Interestingly, there is generally much less multicorn-related activity in cancerous than in control cells and cancer cells contain mostly overphosphorylated, unstable forms of the protease.

Our findings show a fragment of interactions between parts of the controlled proteolysis machinery critical for antigen processing and cell cycle regulation. This web of interactions should be taken into account when anti-cancer drugs target single components of the whole system.

3. Abstract of the poster presented in the 12th Annual Symposium in Cancer Research in San Antonio (July 12, 2002, San Antonio, TX)

Cancer, proteases and proteolytic instability. *Pawel A. Osmulski, Xianzhi Jang, Bingnan Gu, Maria E Gaczynska. Department of Molecular Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78245.

Protein degradation, in concert with protein synthesis, governs the proper execution of metabolic processes in the cell. The large, intracellular proteases like anti-cancer drug target proteasome, a novel protease multicorn, tripeptidyl peptidase II (TPPII) or leucine aminopeptidase (LAP) play a key role among all proteolytic enzymes due to their diverse functions. The proteasome actions are essential for cell cycle regulation, turnover of transcription factors and antigen processing. Inhibition of proteasome leads to cell death and is utilized to kill tumor cells. However, there are strong indications that the other proteases may also constitute valuable anti-cancer drug targets. The postulated duties of multicorn include degradation of cell cycle related factors and, together with TPPII and LAP, further processing of antigenic peptides produced by proteasome. The collaboration of proteasome and other large proteases suggests the existence of a net of functional relationships between the executors of controlled proteolysis. We demonstrate here that proteolytic instability, which manifests in changing the equilibrium between the activities of large cytosolic proteases is one of the signs of neoplastic transformation in human breast cancer MCF7 cells, as compared with non-cancerous MCF10A cells. In the functional proteomics fashion we found prominent changes in activities of the enzymes, in their subunit composition and subcellular distribution. Specifically, we found that there is markedly less proteasomes in nuclei of the cancerous than control cells, and nuclei of cancer and control cells have dramatically different pattern of subunits of a natural proteasome activator. Since proteasome, multicorn, TPPII and LAP all take part in antigen processing, the changes may impair removal of transformed cells by the organism. Probing the role of large proteases in maintaining the proper advance of cell cycle we discovered that the predominantly cytosolic multicorn can be found in the nucleus. Interestingly, both nuclei and cytosol of cancer cells contain mostly overphosphorylated, unstable forms of the protease. We postulate that the nuclear localization of multicorn holds the key for dissecting its role in cell cycle progression.