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HYPERPHOSPHORYLATION OF CALNEXIN, A CHAPERONE PROTEIN, INDUCED BY *CLOSTRIDIUM DIFFICILE* CYTOTOXIN

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Summary: Exposure of McCoy cultured cells to *Clostridium difficile* cytotoxin B or okadaic acid (OA), a potent phosphatase inhibitor, results in similar morphological changes. Using two-dimensional gel electrophoresis, we have detected a protein of approximately 77 kDa, with a pI of 4.5 (termed pp77) which is hyperphosphorylated in both cases. The level of phosphorylation of pp77 is increased by 293% and 35% after treatment with *C. difficile* cytotoxin B or OA, respectively. This protein was identified by microsequencing as calnexin, a protein which exhibits the characteristics of an endoplasmic reticulum (ER) chaperone.

Clostridium difficile is the major cause of antibiotic-associated diarrhea and pseudomembranous colitis in humans. The organism's pathogenicity is associated with its ability to produce two toxins, toxin A (enterotoxin) and toxin B (cytotoxin) (1). However, the mechanism by which the toxins destroy mucosal cells and produce the disease remains unresolved. Toxin B is a potent cytotoxin, 1000 times more cytotoxic than toxin A, which causes rounding and death of most mammalian cell lines *in vitro*.

Previous studies have shown that the rounding of the cells induced by *C. difficile* cytotoxin B is due to a reorganization of the microfilament system of the cytoskeleton (2, 3). However, cytotoxin B has no effect on actin polymerization *in vitro* (4), but seems to affect the phosphorylation status of certain cellular proteins which could subsequently provoke morphological changes (5). Cytotoxin B does not alter protein kinase C nor protein kinase A activities (5). Since no changes in phosphorylation activities were observed, this toxin could therefore be inhibiting phosphatase activities. Interestingly, okadaic acid, an inhibitor of phosphatases 1 and 2A causes rounding of McCoy cultured cells similar to that observed with the cytotoxin.

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In order to investigate the molecular mechanism of the cytotoxicity of *C. difficile* cytotoxin B, we have examined the phosphorylation level of McCoy culture cell proteins in the presence of radiolabeled inorganic phosphate and cytotoxin B or okadaic acid. The radiolabeled cellular extracts were subjected to two-dimensional PAGE and the profiles were compared and analyzed. The identification of the proteins whose phosphorylation status is modified after treatment with cytotoxin is important with respect to their possible role in the molecular mechanism of action of the toxins of *C. difficile*.

Material and methods

Chemicals and cells: Okadaic acid was purchased from Sigma, St Louis, USA. McCoy cells, which are epithelial cells from human synovial membranes, were purchased from Flow, Puteaux, France.

Bacterial strain and toxin purification: *C. difficile* 68750 (Tox +) was isolated in our laboratory from stools obtained from a patient having pseudomembranous colitis. Toxin purification was performed using a fast protein liquid chromatography apparatus by two-step anion exchange chromatography on a Mono Q HR column (Pharmacia LKB, Uppsala, Sweden) as described previously (6).

Preparation of cell extracts: McCoy cells were incubated in phosphate-free medium for 3x10 minutes, then incubated 2 hours at 37°C in the same medium supplemented with 10% fetal calf serum containing 200 µCi ^{32}P orthophosphate. The cells were treated with okadaic acid or *C. difficile* cytotoxin B. The cells were resuspended in 0.3% SDS, 200 mM DTT, 28 mM Tris-HCl, 22 mM Tris-base, pH8 and were then chilled on ice and treated for 8 minutes with DNAse and RNAse at 0°C. The phosphorylated proteins were precipitated with 80% (v/v) ice cold acetone and after centrifugation the air dried pellet was dissolved in 9.9 M urea, 4% Nonidet P-40, 2.2% Millipore optimized carrier ampholytes 3-10, 100 mM DTT. Twenty five µg of each extract was subjected to two-dimensional electrophoresis (10^6 cpm).

Two-dimensional gel electrophoresis and transfers: Two-dimensional PAGE and transfers were performed according to the method of O'Farrell *et al* (7) with modifications as described for the Millipore investigator 2D electrophoresis system (8). After two-dimensional electrophoresis, the radiolabeled crude extracts were transferred onto Immobilon PVDF transfer membrane (Millipore, St Quentin-en-Yvelines, France) and autoradiographed.

Autoradiography scanning: Each autoradiograph was scanned using LKB 2222-020 Ultroscan XL Laser densitometer. Scanning data were analyzed using the LKB 2400 gelscan XL program (Pharmacia LKB, Uppsala, Sweden).

Protein sequencing: Proteins separated by two-dimensional gels were visualized by staining with coomassie blue. The appropriate protein spot (whose phosphorylation was enhanced after treatment with okadaic acid and cytotoxin B) was eluted from a gel and was subjected to tryptic digestion as described by Rosenfeld *et al* (9). The degradation products were purified by HPLC chromatography using a Vydac C18 column (300 Å x 5 µ)(MZ Analyzen, Germany). The samples were run for 20 cycles on a phase sequencer type 477 Å (Applied Biosystems, Foster City, USA) using a standard sequencer protocol. The sequence obtained was compared with the protein sequences in the EMBL/Genbank/DDBJ databank.

Results

A typical silver-stained 2D protein pattern obtained with an untreated McCoy cell extract is presented in figure 1a alongside the autoradiograph (figure 1b). The two-dimensional PAGE pattern of radiolabeled McCoy cell protein extracts after treatment with *C. difficile* cytotoxin B reveals differences in phosphorylation status of several proteins. The profile obtained after okadaic acid treatment is vastly different from that obtained after *C. difficile* treatment (10). However, one protein with a molecular weight of 77 kDa and a pI of 4.5, termed pp77, (figure 1b) is hyperphosphorylated in both cases. Enlargements of the appropriate area of the two-dimensional PAGE autoradiographs are shown in figure 2. In the same region, a second protein with a molecular weight of 80 kDa and a pI of 4.9 (termed pp80) appears to be dephosphorylated uniquely in the presence of okadaic acid. The specific absorbances of these two spots were quantified. Two different extracts were run in duplicate. The total spot area multiplied by average absorbance was measured and adjusted relative to the value obtained for an internal standard reference protein (termed R, figure 1a and 1b).

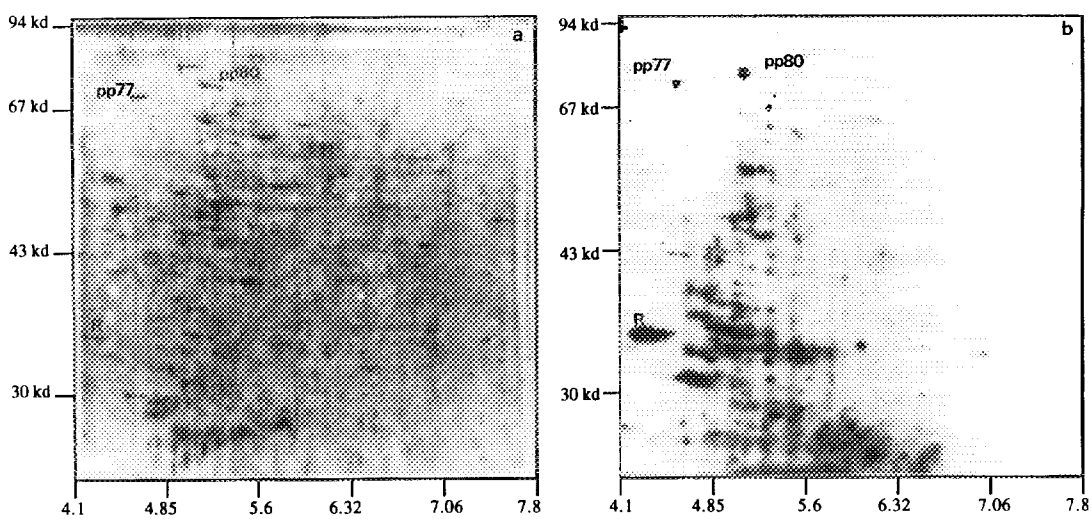

Figure 1a. Silver-staining of McCoy cells separated by two-dimensional gel electrophoresis. Phosphoproteins described in this publication are noted pp77 (calnexin) and pp80 (unidentified). R is the internal standard reference protein.

Figure 1b. Autoradiographs of two-dimensional electrophoresis of radiolabeled phosphoproteins from untreated McCoy cells. After electrophoretic transfer of proteins, the Immobilon PVDF transfer membranes were subjected to autoradiography for 48 hours. Phosphoproteins for which treatment with toxin B or okadaic acid elicited a significant change are noted pp77 and pp80. In the margins, the position of molecular weight markers and the pI gradient are indicated.

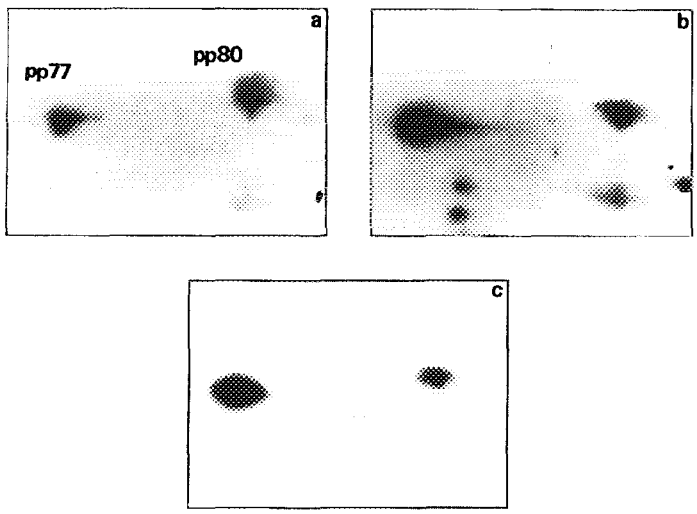

Figure 2. Enlargement of the 80kd area of two-dimensional electrophoresis gels of ^{32}P-labeled McCoy cell extracts. Cells untreated (a),treated with toxin B (b), okadaic acid (c). Unidentified phosphoprotein: pp80, identified phosphoprotein: pp77: calnexin.

A specific absorbance ratio of 35% and 293% was obtained after treatment with okadaic acid or cytotoxin B, respectively, corresponding to an enhancement of incorporation of ^{32}P when compared with the respective spot of the untreated sample i.e. the control. For protein pp80 an absorbance ratio of 1.35% was obtained after treatment with *C. difficile* cytotoxin B, which is insignificant whereas this same protein its dephosphorylated after treatment with OA. (absorbance ratio of – 94.5%) (table I).

The most intriguing question was to identify pp77. After separation by two-dimensional PAGE and transfer to PVDF Immobilon membrane the selected protein spot was subjected to direct NH_2-terminal amino acid sequence analysis. No sequence information could be obtained suggesting blockage of the protein NH_2-terminal, therefore, another approach was adopted. The protein was extracted from a coomassie blue-stained gel, subjected to tryptic digestion, and then sequenced. The initial yield was less than 30 pmol protein but allowed the identification of 14 out of 15 amino acids (KAPVPTGEVYFADXF). A computer search for sequence homologies was performed with the EMBL/Genbank/DDBJ protein sequence databank. This protein was conclusively identified as calnexin (figure 3).

Discussion

C. difficile and *C. sordellii* cytotoxins have been shown to have similar physico-chemical properties and are immunologically related (11). These toxins

Table I: Quantitative analysis of the phosphorylation status of phosphoproteins pp77 and pp80 in response to *C difficile* cytotoxin B or okadaic acid (OA) treatment

	pp77		pp80		R (reference)
	S.A.	change in phosphorylation	S.A.	change in phosphorylation	S.A.
control	6.5		5.6		22.4
toxin B	18.6	270%	4.4	1.7%	17.3
	11.0	316%	2.3	1%	9.1
OA	9.7	36%	0.2	– 97%	24.6
	14.2	34%	0.7	– 92%	36.4

Table I: Autoradiographs were scanned and specific absorbance (S.A.) is defined as spot area (mm2) multiplied by average absorbance of each spot and expressed as arbitary units by the LKB 2400 gelscan XL program (Pharmacia, Uppsala, Sweden). % is defined as the following ratio:

$$\frac{\text{S.A. (toxin B or OA)} \times \frac{\text{S.A. (R control)}}{\text{S.A. (R toxin B or OA)}} - \text{S.A. (control)}}{\text{S.A. (control)}} \times 100$$

No change in ^{32}P incorporation is found at a ratio of 0%. Positive ratio reflects incorporation in ^{32}P whereas negative ratio reflects dephosphorylation. R is the internal standard reference protein, the control corresponds to an untreated sample.

are known to cause a re-organisation of the cytoskeleton in cultured cells, either directly or indirectly (2-5). Since many proteins involved in the regulation of cytoskeletal structure act via phosphorylation or dephosphorylation events, we have used the approach of comparing the two-dimensional PAGE profiles of cellular proteins ^{32}P-phosphorylated *in vivo* in response to these toxins. In this manner we have previously identified, by immunological techniques, a cytoskeletal protein, caldesmon, which is specifically phosphorylated in response to *C. sordellii* cytotoxin (10).

Here we report the identification of a protein (pp77) which is normally phosphorylated and which is hyperphosphorylated in response to *C. difficile*

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                    10
                    KAPVPTGEVYFADXF
                    |||||||||||||:|
TTAPPSSPKVTYKAPVPTGEVYFADSFDRGTLSGWILSKAKK
50        60        70        80        90
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Figure 3. Peptide sequence obtained after tryptic digestion of pp77 and comparison with human calnexin (14).

cytotoxin. This protein was identified as calnexin after microsequencing and comparison with the protein sequence data bank. Investigators had originally described human calnexin as having an apparent molecular weight of 88-90 kDa (12, 13), however sequence data have suggested a molecular weight of 67 kDa (14). Our value of 77 kDa is different due to errors incurred in an estimation from SDS PAGE. We have estimated the pI as 4.5 which compares favorably with pI 4.3 calculated from sequence data. Calnexin is a membrane-bound chaperone protein situated in the endoplasmic reticulum, which is involved in the folding and subunit assembly of nascent proteins. Calnexin is classified as a chaperone protein but is not in fact stress-related and does not appear to have a characteristic nucleotide binding site. Calnexin's role is in protein "quality control", it leads to the retention of misfolded or incompletely folded proteins in the endoplasmic reticulum, and may provide additional time for the correct assembly of complex oligomeric proteins (15). Mammalian calnexin has been shown to be phosphorylated at serine residues by at least one known kinase, casein kinase II (15, 16), and Ca^{2+} is thought to be necessary for substrate binding (17). Interestingly, the activity of *C. difficile* cytotoxin is dependent on intracellular Ca^{2+} (2).

In the light of our results, there are two questions to be addressed, firstly how *C. difficile* cytotoxin alters the phosphorylation of calnexin and secondly, how hyperphosphorylation of calnexin leads to an alteration in cellular morphology typical of cytotoxicity. We have previously shown that *C. difficile* cytotoxin does not act as an activator of protein kinase C nor protein kinase A (5). Alternatively it could be acting as a phosphatase inhibitor. The fact that okadaic acid leads to a similar rounding of cultured cells and we show here that okadaic acid also results in the hyperphosphorylation of calnexin may indicate that *C. difficile* cytotoxin is in fact acting as an inhibitor of phosphatases type 1 and 2A. This aspect will be the focus of further investigation.

Electron microscope studies have revealed that the endoplasmic reticulum becomes swollen in cells treated with *C. difficile* cytotoxin (18, 19). One could envisage that hyperphosphorylation of membrane-bound calnexin could lead to a conformational change resulting on one hand in a disorganisation of the endoplasmic reticulum and on the other hand resulting in a disfunctionment of calnexin's "quality control" mechanism, leading to an accumulation of incompletely and misfolded proteins. Many proteins vital for cellular function and in particular in the regulation of the cytoskeleton could be affected, leading to general cellular disorganisation.

However, one should not ignore the possibility that hyperphosphorylation of calnexin could simply be a secondary effect of cellular intoxication. Either way, our results raise many interesting questions relevant to protein maturation and cytoskeletal regulation and organisation.

References

1. Wolfhagen, M. J. H. M., Torensma, R., Fluit, A. C., and Verhoef, J. (1994) FEMS Microbiol. Rev. **13**, 59-64.
2. Ciesielski-Treska, J., Ulrich, G., Rihn, B., and Aunis, D. (1989)Eur. J. Cell Biol. **48**, 191-202.
3. Ottlinger, M. E., and Lin, S. (1988) Exp. Cell Res. **174**, 215-229.
4. Mitchell, M. J., Laughon, B. E., and Lin, S. (1987) **55**, 1610-1615.
5. Ciesielski-Treska, J., Ulrich, G., Baldacini, O., Monteil, H., and Aunis, D. (1991) Eur. J. Cell Biol. **56**, 68-78.
6. Rihn, B., Bisseret, F., Girardot, R., Scheftel, J. M., Nguyen, V. K., and Monteil, H. (1988) **428**, 408-414.
7. O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P. H. (1977) Cell. **12**, 1133-1142.
8. Patton, W. F., Lopez, M. F., Barry, P., and Skea, W. M. (1992) BioTechniques. **12**, 580-585.
9. Rosenfeld, J.,Capdevielle, J., Guillemot, J. C., and Ferrara, P. (1992) Anal. Biochem. **203**, 173-179.
10. Schué, V., Green, G. A., Girardot, R., and Monteil, H. (1994) Toxicon, in press.
11. Baldacini, O., Girardot, R., Green, G. A., Rihn, B., and Monteil, H.(1992) Toxicon. **30**,129-140.
12. Ahluwalia, N., Bergeron, J. J. M., Wada, I., Degen, E., and Williams, D. B. (1992) J. Biol. Chem. **267**, 10914-10918.
13. Hochstenbach, F., David, V., Watkins, S., and Brenner, M. B. (1992) Proc. Natl. Acad. Sci. **89**, 4734-4738.
14. David, V., Hochstenbach, F., Rajagopalan, S., and Brenner, M. B. (1993) J. Biol. Chem. **268**, 9585-9592.
15. Bergeron, J. J. M., Brenner, M. B., Thomas, D. Y., and Williams, D. B. (1994) Trends Biochem. Sci. **19**, 124-128.
16. Ou, W. J., Thomas, D. Y., Bell, A. W., and Bergeron, J. J. M. (1992) J. Biol. Chem. **267**, 23789-23796.
17. Wada, I., Rindress, D., Cameron, P. H., Ou, W. J., Doherty, J. J., Louvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. M. (1991) J. Biol. Chem. **266**, 19599-19610.
18. Rihn, B., Beck, G., Monteil, H., Lecerf, F., and Girardot, R. (1985) Biol. Cell. **53**, 23-32.
19. v. Eichel-Streiber, C., Warfolomeow, I., Knautz, D., Sauerborn, M., and Hadding, U. (1991) Biochem. Soc. Trans. **19**, 1154-1160.