MCF-7 mammary tumour cells express the myeloid cell differentiation controlling factor, serine protease 3/myeloblastin

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Abstract. Our previous data indicated that HSP27 plays a role in MCF-7 cell differentiation similar to that it has in HL-60 cells. In the latter case, this involves a control of its levels by proteinase 3/myeloblastin (PR3/Mbn), a serine proteinase hitherto considered specific of the myeloid lineage. Having observed that the treatment of MCF-7 cells with the serine protease inhibitor N-tosyl-l-phenylalanine-chloromethyl ketone (TPCK) increased their content in HSP27 and induced them to acquire a secretory phenotype, we undertook this work to test the assumption that an enzyme similar or identical to PR3/Mbn might be expressed in this cell line. The data show that MCF-7 cells exhibited specific immunopositivity for a monoclonal antibody against PR3/Mbn. Western blot analysis of immunoprecipitates from MCF-7 cell extracts, obtained and checked with PR3/Mbn monoclonal antibodies, confirmed the presence of the 35 kDa glycosylated and 29 kDa mature forms of the protein. Finally, Northern blot analysis confirmed the expression of the corresponding mRNA. Together with our data with TPCK, this substantiates our hypothesis that, as in HL-60 cells, regulation of MCF-7 cells differentiation might involve a postranslation control on HSP27 levels by a serine protease.

INTRODUCTION

Induction of HL-60 cells granulocytic differentiation is associated with the down-regulation of an mRNA encoding for a serine protease, initially named 'myeloblastin', and antisense inhibition of the production of this enzyme suffices for provoking proliferation arrest and monocytic differentiation of these cells (Bories *et al.* 1989). Myeloblastin was then identified as protease 3 (PR3) (Goldschmeding *et al.* 1989, Labbaye, Musette & Cayre 1991), an enzyme present in the azurophil granules of human polymorphonuclear leucocytes which is the target of antineutrophil cytoplasmic autoantibodies (c-ANCA) associated to Wegener's granulomatosis (Niles *et al.* 1990), hence its designation as PR3/myeloblastin (PR3/Mbn).

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On the other hand, macrophagic differentiation and the resulting growth arrest of HL-60 promyelocytic leukaemia cells, in response to tetradecanoyl phorbol acetate (TPA), are associated with up-regulation of HSP27 expression (Shakoori *et al.* 1992; Spector *et al.* 1993).

A link between PR3/Mbn and HSP27 was indicated by works showing that the increase in HSP27 content that occurs upon all-*trans*-retinoic acid (tRA)-induced granulocytic differentiation of HL-60 or NB4 promyelocytic leukaemia cells is mirrored by the down-regulation of the serine protease (Shakoori *et al.* 1992; Niles *et al.* 1989). This can be put in relation with the fact that, in accordance with the presence in its gene of sequences encoding serine protease target sites (Hickey *et al.* 1986), HSP27 is sensitive to the action of PR3/Mbn (Spector *et al.* 1995). Together, those data indicated the existence in HL-60 cells of regulatory pathways acting on HL-60 cell differentiation by modulating their content in HSP27 levels, this not only at the transcription or translation level, but also, via PR3/Mbn, at the post-transition level.

There is abundant evidence supporting the concept that, besides its function in resistance to stress (Landry *et al.* 1989; Huot *et al.* 1991; Oesterreich *et al.* 1991), the small heat-shock protein of 2728 kDa and its murine analogue HSP25 also intervene in the control of growth and/or differentiation in various cell types, namely osteoblasts (Shakoori *et al.* 1992) myeloid leukaemia cells (Michishita *et al.* 1991; Shakoori *et al.* 1992; Labbaye *et al.* 1993; Spector *et al.* 1993; Mivechi *et al.* 1994; Minowada & Welch 1995; Spector *et al.* 1995), erythroleukaemia cells (Shakoori *et al.* 1992) or embryonic carcinoma cells (Stahl *et al.* 1992).

A similar situation seems to prevail in MCF-7 mammary tumour cells. Indeed, the production of HSP27 is up-regulated by oestrogen in MCF-7 cells, the action in that case seeming to be at the transcription level (Adams *et al.* 1983; Moretti-Rojas *et al.* 1988). More directly, we recently demonstrated that antisense inhibition of HSP27 production in MCF-7 cells induced their acquisition of a secretory phenotype, associated with a reduction in growth rate, thus mimicking the action of phorbol myristate (TPA), which stimulates HSP27 phosphorylation (Mairesse *et al.* 1996; Horman *et al.* 1997; Horman *et al.* 1999).

Investigating then the hypothesis that a module of regulation similar to that operating in myelocytic cells — i.e. involving control of HSP27 levels by a serine protease—might also operate in MCF-7 cells, we recently showed that a treatment of these cells with the serine protease inhibitor N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK) reproduced the effects of TPA on their growth and morphology (Horman *et al.* 2000).

As a step towards a more direct evaluation of our hypothesis, the present work was aimed at testing the assumption that PR3/Mbn itself hitherto considered specific to the myeloid lineage might in fact be expressed in mammary tumour cells.

Our data indeed confirm this assumption, by showing that MCF-7 mammary tumour cells stain positively with the WGM-2 anti-PR3 monoclonal antibody. Western blot analysis confirmed the presence in these cells of the 35 kDa glycosylated and the 29 kDa mature forms of PR3/Mbn while Northern blot analysis further established the expression of the *PR3/Mbn* gene transcripts.

MATERIALS AND METHODS

Cells

We used human breast cancer cells of the MCF-7 and the MDAMB-231 strains (obtained from Dr G. Leclercq, Institut Jules Bordet, Brussels), respectively, expressing HSP27 at high and low levels. The cells were maintained at 37 °C in a 5% CO₂ atmosphere, in Dulbecco's medium with Eagle's modifications (DMEM) supplemented with 10% foetal bovine serum,

glutamine (3.2 mM), Hepes buffer pH 7 (30 mM) and of penicillin (160 U/ml), streptomycin (150 g/ml) and amphotericin B (fungizone) (GIBCO, Paisley, UK). We also used HL-60 promyelocytic leukaemia cells (obtained from Dr Darzynkiewicz, New York, USA) cultivated in RPMI 1640 medium (GIBCO), with the same supplements as above.

Cytochemical procedures

The clinical interest raised by the identification of myeloblastin with the Wegener's granulomatosis autoantigen PR3 (Goldschmeding *et al.* 1989; Labbaye *et al.* 1991) having made various monoclonal antibodies against this enzyme commercially available, we took benefit of this for directly testing PR3/Mbn expression in our cells.

Immunocytochemical detection of the PR3/Mbn protein was performed on cells fixed for 10 min with 70% ethanol and treated with 50% sheep serum and 10% (weight/volume) of fat-free powdered milk in PBS for blocking non specific binding. The cells were incubated overnight at 4°C with the WGM2 anti-PR3 monoclonal antibody (Csernok *et al.* 1990), diluted 1 : 100 in PBS containing 0.1% bovine serum albumin (PBS-BSA). This antibody was kindly given to us by Dr Elena Csernok. The cells were then washed with PBS-BSA and incubated for one hour at room temperature with a biotinylated antimouse Ig at dilution 1/100, used as binding bridge to biotin-streptavidin-peroxidase preformed complexes added at dilution 1/300 in PBS-BSA. Peroxidase activity was revealed by the mixed chromogenic substrate method of Young as previously described (Horman *et al.* 1997). Negative immunocytochemical controls consisted in replacing the primary antibody by the nonimmune IgG1 isotype at the same concentration. In both cases, the nuclei were lightly counterstained by a 15-s immersion of the slides in Mayer's haematoxylin. Observations were made under a Leitz microscope. Microphotographs were taken using 400 ASA Fujichrome films with fixed exposure time.

Immunoprecipitation and Western blot analysis of PR3/Mbn

Five million cells were put in 1 ml of lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and a proteinase inhibitor mixture (aprotinin, pepstatin, leupeptin, pefabloc, EDTA). Cell lysate was mixed with 50 µl of the homogeneous protein G-agarose suspension (Boehringer Mannheim, Mannheim, Germany) and incubated for 3 h at 4°C on a rocking platform. Supernatant was recovered by centrifugation at 12000 *g* for 1 min and incubated for 1 h at 4°C, after gentle mixing with the monoclonal antibody MCPR3-2 (Sun *et al.* 1998), generously provided by Dr Ulrich Specks. Fifty µl of protein G-Sepharose beads were then added to the mixture and incubated overnight at 4°C with gentle shaking. Protein G-bound immune complexes were then washed, suspended in 50 µl of SDS-sample buffer, boiled for 3 min, and submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

For Western blot analysis, the electrophoregrams were transferred onto a Polyscreen PVDF transfer membrane (NEN Life Science Products, Zaventem, Belgium) and checked with two antibodies recognizing different, nonoverlapping epitopes (Van Der Geld, Limburg & Kallenberg 1999), used at a 1 : 200 dilution, namely the MCPR3-2 monoclonal antibody or the CLB-12.8 monoclonal antibody described by Goldschmeding *et al.* (1989), a kind gift from Dr Elena Csernok.

The immune complexes were detected with a biotinylated antimouse Ig (diluted 1 : 200) used as a binding bridge to preformed biotin-streptavidin-peroxidase complexes. Peroxidase activity was revealed by enhanced chemiluminescence (ECL) using the Renaissance Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products).

Northern blot detection of PR3/Mbn transcripts

The PR3/Mbn cDNA probe was obtained from human HL-60 cells by reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using the High Pure RNA Isolation Kit (Boehringer Mannheim) according to the manufacturer's instructions. Two primers, corresponding to the 19 first and the 21 last nucleotides of the human PR3/Mbn coding sequence (Labbaye, Musette & Cayre 1991) were designed and used to amplify the 768 bp full-length product

(sense: 5' ATGGCTCACCGGCCCCCA;

antisense: 5'TCAGGGGCGGCCCTTGGCCTC).

In a 50- μ l reaction volume, the following components were added: 10 μ l of RT-PCR buffer (5X stock), 2.5 μ l of DTT solution (100 mM), 4 μ l of dNTP mixture (10 mM stock), 0.5 μ g of each primer, 1 μ g of total RNA, 1 μ l of enzyme Mix (Expand High Fidelity enzyme mix and reverse transcriptase AMV, in storage buffer) and water. Just before adding the enzyme mix, RNA was linearized by 5 min heating at 80 °C and 1 min cooling on ice. The solution was covered with mineral oil and incubated in a Biometra thermal cycler using 40 cycles of 1 min 95 °C and 2 min 72 °C primer annealing temperature and polymerase extension temperature. At the end of the cycling phase, a further extension period of 10 min at 72 °C was performed.

The cDNA reaction as well as the PCR were performed with the Titan™ One Tube RT-PCR System (Boehringer Mannheim), without addition of reagents between cDNA synthesis and PCR. The complete PCR products were electrophoresed on a 1.4% (W/V) agarose gel, and were visualized by staining with ethidium bromide before being extracted from TAE agarose gels with the Agarose Gel DNA Extraction Kit (Boehringer Mannheim). Treatment of the probe with the restriction enzymes BamH1 and PstI generated fragments with the expected size, based on the published sequence of PR3/Mbn (Labbaye *et al.* 1991), thus confirming correct amplification of the full-length PR3/Mbn sequence. The probe was labelled with biotin, using therefore the Random Primer Biotin Labelling Kit™ (NEN Life Science Products).

For Northern blot analysis, total cellular RNA was isolated as above, using the High Pure RNA Isolation Kit (Boehringer Mannheim). RNA preparations were denatured and processed as described by Kruys, Thompson & Beutler (1993). All RNA blots contained 10 g of total RNA per lane. Ethidium bromide-stained 28 s and 18 s RNA (rRNA) were used as reference for RNA quantities transferred on the filter. The filters (GeneScreen Hybridization Transfer Membrane) were hybridized to 20 ng/ml of the biotin-labelled-full-length PR3/Mbn cDNA probe. Peroxidase activity was revealed by ECL using the Renaissance enhanced luminol chemiluminescent reagent for nucleic acids (NEN Life Science Products).

RESULTS

Immunological detection of PR3/Mbn

Immunocytochemical staining of MCF-7 cells with the WGM-2 monoclonal antibody against PR3/Mbn revealed a dense cytoplasmic and nuclear immunopositivity in most of the cells and a weaker one in about 10% of the population (Fig. 1). Submitted to the same immunocytochemical procedure, MDAMB-231 cells exhibited similar immunopositivity, whereas HL-60 cells stained less intensely (not illustrated). The isotype specificity controls always remained negative, even at an IgG concentration twice that used for the primary antibody (Fig. 1).

That this positivity reflected the actual presence of PR3/Mbn products in MCF-7 cells was then checked by Western immunoblot analysis of the immunoprecipitate obtained by treating cell extracts with the MCPR3-2 monoclonal antibody.

Figure 1. (a) Cytochemical detection of PR3/Mbn in MCF-7 cells using the WGM2 anti-PR3 monoclonal antibody; (b) nonimmune isotype control (magnification: $\times 640$). Immune complexes were detected by bridging biotinylated goat antimouse antibody to preformed streptavidin-biotin-peroxidase complexes, in turn revealed by reaction with the peroxidase substrate diaminobenzidine; nuclei lightly counterstained with haematoxylin.

A series of four bands, not detected in the isotype control, were revealed in the blots by the MCPR3-2 antibody (Fig. 2). Their position corresponded to peptides with an apparent molecular mass of about 34 (the faintest band) 29, 26 and 24 kDa (the most intensely chemiluminescent band). The CLB-12.8 anti-PR3 antibody gave less intense reaction and only detected the product of 24 kDa (Fig. 2).

A product or group of products of about 24 kDa also appeared in Western blots from human recombinant PR3 illustrating reports by other authors (Witko-Sarsat *et al.* 1996), when the cell lysate had been submitted to further fractionation before loading on the gel,

Figure 2. Western blot analysis on Polyscreen PVDF blots from one-dimensional SDS-PAGE electrophoregrams of the immunoprecipitate obtained by reacting MCF-7 cells lysates with the MCPR3-2 mouse monoclonal antibody against PR3/Mbn, non reducing conditions being maintained throughout the procedure (see Materials and Methods for details). *Lane 1:* isotype control; *lane 2:* immunoreaction with CLB-12.8; *lane 3:* immunoreaction with MCPR3-2.

indicating that this reflected partial degradation of the native protein during the ensuing delay between obtention of the extract and loading on the gels. As a mixture of antiproteases was present in our experiments throughout the immunoprecipitation process, the 24–26 kDa PR3/Mbn fragments that we detected may reflect partial degradation of the protein *in situ*, in the living cells.

Finally, the heaviest products that we detected as two lightly luminescent bands correspond by their position in the gel to the products detected in extracts from U937 cells by other authors, who identified them as the 35 kDa glycosylated pro-PR3 and the 29 kDa mature protein into which it is converted by triple proteolytic cleavage and trimming of oligosaccharides (Rao *et al.* 1996).

Northern blot detection of PR3/Mbn transcripts

The PR3/Mbn cDNA probe detected one single band in the blots from electrophoregrams of total RNA fraction from HL-60 cells, used as positive reference (Fig. 3). Its position (relative to that of the 18 s and 28 s ribosomal RNA) was that expected for the fully processed PR3/Mbn transcripts (Labbaye *et al.* 1991). In agreement with reports showing that dimethylsulfoxide (DMSO) induced differentiation of these cells is accompanied by the down-regulation of PR3/Mbn (Shakoori *et al.* 1992; Labbaye *et al.* 1993), the intensity of this band was markedly decreased in extracts from HL-60 cells that had been treated with 1.4% DMSO (not illustrated). The PR3/Mbn probe detected the same band in RNA preparations obtained from MCF-7 cells (Fig. 3), thus confirming the production of the PR3/Mbn transcripts by these mammary tumour cells.

DISCUSSION

PR3/Mbn expression is generally considered to be restricted in cells of the myeloid lineage (Csernok *et al.* 1990; Witko-Sarsat *et al.* 1996). Immunocytochemical studies indeed revealed the presence of this proteinase in neutrophil granulocytes, mastocytes and a subset of monocytes in peripheral blood, as well as in the monocytic line THP-1 and, as mentioned

Figure 3. Representative Northern blot detection of *PR3/Mbn* gene transcripts in HL-60 or MCF-7 cells, as indicated. (Position of 28 S and 18 S RNA indicated on upper part is based on ethidium bromide staining of the gel).

above, in the promyelocytic line HL-60 (Muller-Bérat *et al.* 1994; Dengler *et al.* 1995). Accordingly, other studies found it expressed in normal myeloid cells and in myelogenous leukaemia cells present in human peripheral blood or in bone marrow aspirates, but not in cells from lymphoid, erythroid or megakaryocytic lineage (Gupta, Niles & Arnaout 1990; Sturrock *et al.* 1992). This led us to propose PR3/Mbn as a specific marker for discriminating between myeloid and lymphoid leukaemia (Dengler *et al.* 1995), which is not invalidated by our observation with MCF-7 cells.

However, little attempt had been previously made to check for the possible expression of this serine protease in cells from solid tissue origin. In one of the above mentioned immunohistochemical studies, diverse solid tissues, normal and malignant, were examined—including samples from lung, stomach, colon, spleen, tonsil, blood vessels and nasal mucosa—and positivity was detected only in the granulocytes present in the material (Braun *et al.* 1991). One group reported on immunopositivity for a purified anti-PR3 antibody in human endothelial cells (Mayet *et al.* 1993) and, more recently, in renal tubular cells (Schwartz *et al.* 1997) in culture, yet without establishing by Western blot the identity of the product thus recognized, the presence of the corresponding mRNA transcripts being confirmed in the latter case only.

Our demonstration that MCF-7 cells do express PR3/Mbn raises questions from a fundamental point of view, as well as about the possible clinical implications of this observation. In the latter respect, the finding that cells from nonhematopoietic lineage can express PR3/Mbn may open new ways in our thinking about the still elusive role that, as target to cANCA autoantibodies associated with Wegener's granulomatosis, this serine proteinase—hitherto considered specific to the myeloid lineage—plays in the overall clinical picture of this disease.

Another clinical aspect concerns the possible implication of PR3/Mbn expression in the malignant behaviour of mammary cells. Preliminary assays performed on freshly obtained biopsy samples allowed us to detect the presence of mRNA transcripts of the *PR3/Mbn* gene

in two human mammary tumours (Horman, unpublished), thus indicating the possible relevance of our finding on cells in culture to the *in vivo* conditions.

From a fundamental point of view, and more directly relating to the main aim of this work, the fact that MCF-7 cells do express PR3/Mbn fulfils a basic requirement of our hypothesis that a post-translation control by PR3/Mbn on HSP27 expression might intervene in regulating the differentiation of these cells, in a way similar to that described in HL-60 cells.

This is indeed in line with our recent demonstration that a treatment of MCF-7 cells with the serine protease inhibitor TPCK resulted in increasing their content in native and phosphorylated HSP27 and induced their acquisition of a secretory phenotype (Horman *et al.* 2000) and suggests that this reflected an action of TPCK on PR3/Mbn itself. As this would be on the *activity*—and not on the *level*—of the enzyme, this is not readily amenable to direct evaluation. Indeed, even assuming feasibility of the assay, one would not be ensured that working with cell extracts, *in vitro*, would actually reproduce the *in situ* situation regarding PR3/Mbn activity and the conditions in which this is influenced by TPCK in the intact cells.

Directly acting on PR3/Mbn expression, e.g. resorting to inhibition of PR3/Mbn production by a transfected *antisense PR3* sequence—would represent a more direct and reliable approach to examine functional relationships between PR3/Mbn and HSP27 in MCF-7 cells and the role this may play in their differentiation.

The possibility must also be envisaged that PR3/Mbn might affect other functional proteins than HSP27. In this respect, a recent work showed that, like HSP27 (Spector *et al.* 1995), the Sp1 transcription factor is a substrate of PR3/Mbn (Rao *et al.* 1998). These authors also showed that Sp1 is truncated by this enzyme in undifferentiated HL-60 cells, and they noted that the down-regulation of PR3/Mbn—which is associated with differentiation in these cells—would thus easily account for the expression of a new repertoire of genes (Rao *et al.* 1998).

Interestingly, this seems to bring us back to the role of HSP27 in MCF-7 cells. It was indeed demonstrated (and this precisely in MCF-7 cells) that the Sp1 transcription factor forms with the oestrogen receptor (ER) an Sp1/ER complex which is involved in the estradiol-induced expression of the *hsp27* gene (Porter *et al.* 1996). It was also shown that expression of the small heat shock protein is up-regulated by oestrogen in osteoblasts (Cooper & Uoshima 1994) and in MCF-7 cells (Adams *et al.* 1983; Moretti-Rojas *et al.* 1988; Mairesse *et al.* 1996). In the latter case, this involves an action at the transcription level (Moretti-Rojas *et al.* 1988), suggesting the possible implication of PR3/Mbn in this effect.

As indicated above, whereas this is supported by our previous observation that TPCK treatment of MCF-7 cells resulted in increasing their HSP27 content (Horman *et al.* 2000), more direct evidence, based on antisense inhibition of PR3/Mbn production, would help in clarifying this point.

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