PTHRP [67-86] regulates the expression of stress proteins in breast cancer cells inducing modifications in urokinase-plasminogen activator and MMP-1 expression

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Summary
It was previously reported that a midregion domain of parathyroid hormone-related protein (PTHRP), that is, [67-86]-amide, is able to restrain growth and promote matrigel penetration by the 8701-BC cell line, derived from a biopsy fragment of a primary ductal infiltrating carcinoma of the human breast, and that cell invasion in vitro is drastically impaired by inactivation of urokinase-plasminogen activator (uPa). In this study we started a more detailed investigation of the possible effects on gene expression arising from the interaction between PTHrP [67-86]-amide and 8701-BC breast cancer cells by a combination of conventional-, differential display- and semi-quantitative multiplex-polymerase chain reaction (PCR) assays. We present here the first evidence that the upregulation of some stress-related genes, most noticeably heat shock factor binding protein-1 (hsbp1) and heat shock protein 90 (hsp-90), is involved in the acquisition of an in vitro more invasive phenotype by cells treated with midregion PTHrP. This is conceivably accomplished by sequestering and inactivating heat shock factor-1 (hsf1) which is able to recognize Ets transcription-factor-binding sites present in some gene promoters, such as those of uPa and matrix metalloprotease-1 (MMP-1). In fact, our data show that incubation of PTHrP [67-86]-amide-treated cells with either antisense hsbp1-oligonucleotide or geldanamycin, an hsp90-inactivating antibiotic, results in downregulation of uPa and upregulation of MMP-1, and in a prominent inhibition of cell invasion in matrigel-containing Transwell chambers. Alternatively, incubation of untreated 8701-BC cells with quercetin, a flavonoid known to decrease the amount of free hsf1, is found to induce upregulation of uPa and downregulation of MMP-1, and an increase of matrigel invasion by cells, thus providing further supporting data of the involvement of hsf unavailability on the modulation of uPa and MMP-1 expression and on cell invasive behaviour. These studies confirm a previous postulate that over-secretion of uPa, rather than of other extracellular proteases, is a primary condition for the increase of invasive activity triggered by PTHrP [67-86]-amide in vitro, and support a role for midregion forms of PTHrP in potentially affecting pathological mammary growth and differentiation. They also identify two new key protagonists in the complex scenario of breast tumor cell invasiveness in vitro, that is, hsbp1 and hsp90, which deserve further and more extensive studies as potential and attractive molecular targets for anti-breast cancer treatments.

Key words: PTHrP, Stress proteins, Protease, Breast cancer cells, Gene expression, Invasion

Introduction
The parathyroid hormone-related peptide (PTHRP), classically regarded as the mediator of the humoral hypercalcemia of malignancy syndrome, is the product of a gene spanning more than 15 kb of genomic DNA and exhibiting a complex organization in humans, where it generates multiple mRNA variants through alternative splicing events and utilization of different transcriptional start-sites. Translation of PTHrP mRNAs produces three protein isoforms of either 139, 141 or 173 aminoacids with distinct C-terminals, displaying sequence homology with PTH at extreme N-terminus which allows the binding to the same G protein-linked receptor (reviewed by Philbrick et al., 1996). On the basis of its primary structure, it is generally acknowledged that PTHrP may be a polyhormone undergoing a possibly tissue-specific proteolytic processing into smaller bioactive forms, by members of the subtilisin family of proteases (e.g. furin, PC1/3, PC2, PACE4, PC8) (Guise et al., 2002); some of these secretory species have been either immunodetected or isolated from patients’ serum or conditioned media of normal and neoplastic cells (e.g. Burtis, 1992; Soifer et al., 1992; Yang et al., 1994; Wu et al., 1996). Apart from its classical PTH-like effect, PTHrP was proven to exhibit a diverse array of local autocrine/paracrine growth factor-like roles imparted by its non-N terminal moieties in several model systems (e.g. Fenton et al., 1994; Orloff et al., 1996; Whitfield et al., 1996; Valin et al., 1997; Luparello et al., 2001; De Miguel et al., 2001). Interestingly, PTHrP has also been proven to have intracrine action (Henderson et al., 1995) via a mid-region...
nuclear targeting sequence which allows nuclear/nucleolar accumulation of expressed PTHrP mediated by the importin β/Ran protein system, thus by-passing the requirement for binding to cell surface receptors (Lam et al., 1999; Lam et al., 2000); moreover, Aarts et al. (Aarts et al., 1999; Aarts et al., 2001) have demonstrated the RNA-binding ability of PTHrP and its involvement in ribosome biogenesis, hence suggesting a role for this protein in intracellular events, such as RNA transcription and/or processing, that influence cell cycle progression (Massfelder et al., 1997). Noteworthy, evidence exist that PTHrP is actually involved in the control of cell proliferation, differentiation and survival of cartilage and bone cells, its gene being a downstream target for ras and src and an upstream element of the Bcl-2 and c-fos signaling pathways (Li and Drucker, 1994; Amling et al., 1997; McAuley et al., 1997).

In the mammary gland, PTHrP acts as a critical regulatory factor involved in epithelial-mesenchymal cell interactions (for reviews, see Wyssolmerski and Stewart, 1998; Dunbar and Wysolmerski, 1999) and its expression is essential for branching morphogenesis and sexual dimorphism during organ development (Wyssolmerski et al., 1998; Dunbar et al., 1999). In addition, some of us have produced data demonstrating that different PTHrP domains are also biologically active on breast tumour cells (Luparello et al., 1995; Luparello et al., 1997a; Luparello et al., 2001), and that PTHrP expression by cells is drastically modulated by modifications of the culture microenvironment (Luparello et al., 1999; Luparello et al., 2000a). In particular, a mid-region PTHrP domain, i.e. [67-86]-amide, administered at 1 nM concentration was found able to restrain growth and promote invasion through artificial basement membrane (matrigel) by the 8701-BC cell line, derived from a biopsy fragment of a primary ductal infiltrating carcinoma (DIC) (Minafra et al., 1989), and a highly tumorigenic clonal line of the same cells (Luparello et al., 1997b). Matrigel penetration was drastically impaired by treatment of both cell lines with anti-urokinase plasminogen activator (uPa) antibodies blocking uPa-uPa receptor interaction; although to a lesser extent, tissue inhibitor of metalloprotease (TIMP)-1 was also active in reducing the invasive motility of only 8701-BC cells. By contrast, trypsin- and cystein-protease inhibitors were poorly active in restraining cell invasion (Luparello et al., 1995; Luparello et al., 1997a).

The observation of the invasion-promoting role played by PTHrP [67-86]-amide prompted a more detailed study of the possible effects on gene expression arising from its interaction with breast cancer cells. In this study we demonstrate that treatment of 8701-BC cells with this midregion PTHrP peptide is linked to the upregulation of heat shock factor-binding protein 1 (hsbp1), coding for a factor known to interact with heat shock factor (hsf)1 trimers and negatively regulate hsf1 activity (Satyal et al., 1998; Cotto and Morimoto, 1999), and of some members of heat shock protein (hsp) family, noticeably hsp90α and β, and that such over-expression is involved in the modulation of uPa- and matrix metalloprotease-1 (MMP-1); interstitial collagenase) gene expression and of the invasive behaviour in vitro of 8701-BC breast cancer cells.

Materials and Methods
Cell culture and treatments
The tumor cell line 8701-BC, derived from a biopsy fragment of a primary DIC of the breast (Minafra et al., 1989) was routinely cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and antibiotics (100 U penicillin and 100 μg streptomycin/ml) at 37°C in a 3% CO2 atmosphere.

Treatment with PTHrP [67-86]-amide was performed as described by Luparello et al. (Luparello et al., 1995; Luparello et al., 1997a). Cells were plated at 2.5 × 104 cells/cm² in FCS-containing RPMI 1640 medium and, after overnight incubation to allow adhesion, cultured in serum-free medium for an additional 24 hours; subsequently, serum-free RPMI 1640 medium supplemented with 1 nM PTHrP [67-86]-amide (Peninsula, Belmont, CA) was added to the cultures. After 24 hours, fresh medium with the same peptide supplement was added and the cells were incubated for a further 24 hours, and stored at –80°C until submitted to RNA extraction. Control assays were performed in the absence of PTHrP [67-86]-amide.

Treatment with hspb1-antisense oligo (asODN) was performed as described by Ziegler et al. (Ziegler et al., 1999). Essentially, cells were incubated for 24 hours with 600 nM of phosphorothioate 5′-GTGATGTCTCAGACC-3′ ODN (MWG Biotech, Ebersberg, Germany), complementary to bases 18-32 of hspb1 mRNA (Acc. nr. AF068754), complexed with 18.6 μl lipofectin/ml (Life Technologies, Gaithersburg, MD) and added to the plain or PTHrP-containing medium. Control assays were performed in the presence of phosphorothioate 5′-ACTACAGGTTGCTC-3′ ODN (scrambled-asODN), whose sequence was not found in EMBL-EBI human DNA database, after homology search with the Fast3 software available at http://www.ebi.ac.uk/fasta33/. The RNAdraw software (Matzura and Wennborg, 1996) was utilized to predict the secondary structure of hspb1 mRNA.

Treatment with geldanamycin was performed according to Knowlton and Sun (Knowlton and Sun, 2001). Essentially, cells were incubated for 24 hours with 1 μg geldanamycin/ml (Alomone Labs, Jerusalem, Israel), added to the plain or PTHrP-containing medium. Control assays were performed with dimethyl sulfoxide (DMSO) vector only.

Treatment with 100 μM quercetin (USB, Cleveland, OH) was performed for 24 hours on 8701-BC cells cultured in unsupplemented RPMI 1640 medium (Hosokawa et al., 1992). Control assays were performed with DMSO vector only.

RNA extraction and reverse transcription
Isolation of either total or poly(A)+ mRNA from control and treated 8701-BC cells was performed with TriPure reagent (Roche, Mannheim, Germany) or oligo(dT)25-tailed magnetic beads (mRNA DIRECT kit, Dynal, Oslo, Norway), respectively, following manufacturers’ instructions. Before the reverse transcription, the RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) for 30 minutes at 37°C. The cDNAs were synthesized using M-MLV RNase-H– point mutant reverse transcriptase (Promega), 50 U RNase inhibitor (Promega) and 0.5 μM each of dNTPs; reverse transcription was performed for 60 minutes at 37°C, followed by RNase H treatment for 20 minutes at 37°C.

Conventional qualitative polymerase chain reaction (PCR)
PCR analysis was performed using 2.5 μM of appropriate sense and antisense primers (Table 1) obtained from MWG Biotech, 1 U RedTaq DNA polymerase/μl (Sigma, St Louis, MO), 250 μM each of dNTPs, and 1 μl of the cDNA template obtained from total RNA. The thermal cycle used was a denaturation step of 94°C for 3 minutes, followed by 35-45 cycles of 94°C for 1 minute, the appropriate annealing temperature for 1 minute, and 72°C for 1 minute. A final extension of the product was performed for 10 minutes at 72°C. PCR products were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light.
Table 1. Sequence of primers used for PCR amplification

| Transcript detected | Oligonucleotides (5’→3’) | Product size (bp) | Reference |
|---------------------|--------------------------|-------------------|-----------|
| hsp1                | TGATCTTGGAAAAAGATATCGCG | 168               | *         |
|                     | TTAGTCTGAAAAAAGCCATTCC  |                   |           |
|                     | ACCCGATCCACACACCGA      |                   |           |
| hsp10               | AGTTTCTTCACTTTTACCCGA   | 133               | *         |
|                     | ATCCAGAATTGCGCATTCC     |                   |           |
|                     | GAGTTAGAACATGGCCACCT    |                   |           |
| hsp27               | ACGCCGTTGGAGATCACC      | 350               | Wang et al., 1999 |
|                     | CAAAGAACACACAGGTTGCG    |                   |           |
| hsp60               | ATCCAGAATTGCGCATTCC     | 306               | Wang et al., 1999 |
|                     | GAGTTAGAACATGGCCACCT    |                   |           |
| hsp70               | TCTCCGTTCGAGCCCCCAATC   | 558               | Wang et al., 1999 |
|                     | CGTIGAGCCCGGAGATGACA    |                   |           |
| hsc70               | AAGTGTCAATGAACAGGACC    | 570               | Wang et al., 1999 |
|                     | TTGGCTCAACTCGAGCA       |                   |           |
| mthsp75             | TGGCCAGTTGAGAGTAAAG     | 524               | Wang et al., 1999 |
|                     | AGCAAGTACCTTGTACTTG     |                   |           |
| grp78               | GATAATACCAACAACTTTAC    | 577               | Wang et al., 1999 |
|                     | GTATCTCTTCCACAGTITGG    |                   |           |
|                     | TACCAAGCATCTTATAGTA     |                   |           |
| hsp90α              | AAGAGGTGGAGAGTGTGACA    | 625               | Wang et al., 1999 |
|                     | AAAGAGTGGAGAGGGAATG     |                   |           |
| hsp90β              | CCACGCAACAGGAAATGGTG    | 641               | Wang et al., 1999 |
|                     | TATCAGGCTACACTTATAGTA   |                   |           |
| hsf1                | ATGGAAGCGTGCACCTCTGC    | 120               | *         |
|                     | AATGGTCTGACACCTTCTG     |                   |           |
| hsf2                | ATGAAAGCATGTCACAGCTG    | 246               | *         |
|                     | CCGGCAATTTCCAGGACATATG  |                   |           |
| uPA                 | AAGATTGACACCTATCGG      | 474               | Noguchi-Takino et al., 1996 |
|                     | ATCGCTTCAACACAGTCCAT    |                   |           |
| MMP-1               | GCATTCTGACCATATGGAGCC   | 120               | *         |
|                     | ATACACTTCTCCCCGAATCGT   |                   |           |
| β-actin             | GTGGGACGCCTCCAGACCA     | 548               | Luparello et al., 1993 |
|                     | TTCCTTGAATGTCAGCCAGATTT |                   |           |

*The primers for hsp1 (accession number AF068754), hsp10 (accession number 6996445), hsf1 (accession number M64673), hsf2 (accession number M65217) and MMP-1 (accession number NM_002421) were chosen using the Primer Selection software, available online at http://alces.med.umn.edu/VGC.html, and the identity of the amplification products was checked by sequencing.

Differential display-polymerase chain reaction (DD-PCR)

For differential display analysis, DD-PCR experiments were performed using the arbitrary 10-mer primers designed by Sokolov and Prockop (Sokolov and Prockop, 1994), in combinations of two. The PCR amplification was performed in an UNO Thermoblock (Biometra, Göttingen, Germany) using 25 pmols of each of two primers, 1-2 μl of the cDNA template obtained from mRNA and 3.6 U of AmpliTaq DNA Polymerase, Stoffel fragment (Perkin Elmer), as recommended by Doss (Doss, 1996), in 50 μl of the appropriate reaction mixture. The thermal cycle used was a denaturation step of 94.5°C for 3 minutes, followed by 45 cycles of 94.5°C for 1 minute, 34°C for 1 minute, 72°C for 1 minute and a final extension of the product for 10 minutes at 72°C.

After PCR amplification, 8 μl of the amplification products were electrophoresized in a non-denaturing 6% polyacrylamide gel in a sequencing apparatus (2423 cm0.4 mm; Sequi-Gen, Bio-Rad, Richmond, CA) at constant 55 W and the bands of interest carefully scratched from the gel with a sterile syringe needle and used as template for PCR amplifications performed as described before. Several cycles of amplification and electrophoresis were repeated until a single pure band was visualized in the gel and eluted using Ultrafree DA filter columns (Millipore, Bedford, MA).

The purified PCR products were subsequently cloned using the pGEM-T Easy vector system (Promega) and JM109 competent cells, high efficiency (Promega), as described (Sirchia et al., 2001); the sequence of the inserts contained in the recombinant plasmid DNA, isolated with High Pure Plasmid Isolation kit (Roche), was determined by MWG Biotech sequencing service. DNA sequence similarity was searched with the BLAST algorithm (Altschul et al., 1990) available online at http://www.ncbi.nlm.nih.gov.

Semi-quantitative ‘multiplex’ polymerase-chain reaction (SM-PCR)

For SM-PCR we followed the protocol of Spencer and Christensen (Spencer and Christensen, 1999) with minor modifications. Essentially, the cDNA species of interest was co-amplified with β-actin cDNA (see Table 1 for primer sequences) over a range of cycles, followed by 2% agarose electrophoresis and ethidium bromide stain. Cycle profile was a denaturation step of 94.5°C for 3 minutes, followed by cycles of 94.5°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute and a final extension of the product for 5 minutes at 72°C. Cycles were limited to the minimum necessary for detection and the intensities of the bands of interest, evaluated with SigmaScan software (SPSS), were normalized for those of β-actin, and plotted as a function of cycle number. Exponential regression equations fitted to the curves were used to calculate the number of cycles necessary to reach a normalized intensity threshold value=1 for each sample. The relative difference in abundance between two samples was taken as 2^ΔΔCt, where ΔΔCt is the difference between the numbers of cycles required by the samples to reach the threshold. Two different RNA preparations from each experimental condition were pooled to make the differences, if any, between the expression levels more significant.
Western blot

8701-BC cells were seeded in FCS-containing RPMI 1640 medium, submitted to the treatments described in the previous paragraph, scraped in 0.1% EDTA-containing PBS and counted with a Bürker chamber. Aliquots of 10^6 cells were spun down in PBS and lysed with 100 μl of pre-warmed lysis buffer (40 mM Tris-HCl, pH 6.8, containing 1% sodium dodecyl sulphate, 1% glycerol, 1% β-mercaptoethanol, and 0.001% bromophenol blue). Aliquots of 20 μl of the extracts were subjected to SDS-PAGE (7.5% acrylamide) and blotted to nitrocellulose filters by using a Bio-Rad transfer apparatus. Following the blot, the protein gel was stained with Coomassie Blue. After blocking for 1 hour with Tris-buffered saline/0.05% Tween-20 (TBS-T) containing 5% nonfat dry milk at room temperature, filters were incubated for 1 hour with either anti-hsp90 from rat (Calbiochem, San Diego, CA; final dilution 1:500), anti-hsf1 (Alexis Biochemicals; final dilution 1:10,000) or anti-hsf2 (US Biologicals, Swampscott, MA; final dilution 1:200) in TBS-T containing 1% nonfat dry milk. After being washed six times for 5 minutes each with TBS-T, the filters were incubated for 1 hour with the appropriate peroxidase-conjugated secondary antibody dissolved in TBS-T containing 1% nonfat dry milk and washed six times for 5 minutes each with TBS-T, prior to detection by the SuperSignal Chemiluminescent substrate (Pierce), following manufacturer’s recommendations.

Viability and proliferation assay

The survival and growth behaviour of 8701-BC cells in response to the different treatments was evaluated using the colorimetric CellTiter 96 AQueous One Solution Cell Proliferation assay (Promega), in which the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] compound is reduced into soluble colored formazan by metabolically active cells. According to manufacturers’ instructions, 8701-BC cells were seeded in FCS-containing RPMI-1640, submitted to the treatments described elsewhere in this paper, and incubated, at the end of treatment, with CellTiter reagent for 1 hour in a humidified incubator. The absorbance of formazan produced was recorded at 490 nm.

Invasion assay

Cell invasive behavior was evaluated using Transwell plates (Costar, Cambridge, MA) with 8 μm insert diameter- and 8 μm pore diameter-filters, separating an upper compartment of 600 μl and a lower compartment of 100 μl, following an already-published protocol (Luparello et al., 1995; Luparello et al., 1997a). The filters were coated with 8 μg of Matrigel, a reconstituted basement membrane matrix from EHS sarcoma (Collaborative Res., Bedford, MA); trypsinized 8701-BC cells were washed first with 10% FCS-RPMI 1640 medium for enzyme inactivation and then twice with unsupplemented medium, and 150,000 cells were seeded in the upper compartment of the chamber. Following the blot, the protein gel was stained with Coomassie Blue. After blocking for 1 hour with Tris-buffered saline/0.05% Tween-20 (TBS-T) containing 5% nonfat dry milk at room temperature, filters were incubated for 1 hour with the appropriate peroxidase-conjugated secondary antibody dissolved in TBS-T containing 1% nonfat dry milk and washed six times for 5 minutes each with TBS-T, prior to detection by the SuperSignal Chemiluminescent substrate (Pierce), following manufacturer’s recommendations.

Results

PTHrP [67-86]-amide upregulates hsbp1 in 8701-BC cells

In order to identify target genes for mid-region PTHrP treatment, we first submitted cDNA preparations from control and treated 8701-BC cells to DD-PCR as described. Among the several differentially displayed bands present in the gel, we focused our attention on an amplification band of approximately 180 bp, obtained utilizing the BS70/BST3 combination of arbitrary primers (Sokolov and Prockop, 1994), which was strongly stained in the electrophoretic lane corresponding to the cDNA preparation from treated cells (Fig. 1A). The differentially displayed cDNA fragment was purified by excision from the gel and several cycles of PCR and electrophoresis, cloned and submitted to automated sequencing. Using BLASTN 2.0.12, homology (score=207 bits, expect=6e-51) was found in the non-redundant nucleic acid sequence (nr-nt) database between the sequence obtained and that of region 55-200 of the coding sequence for Homo sapiens hsbp1 (Accession number AF068754) as deposited by Satyal et al. (Satyal et al., 1998).

We then checked the differential expression of hsbp1 by PCR amplification in the presence of two primers specific for this cDNA, designed using the Primer Selection software available online. As shown in Fig. 1B, in a preliminary assay we found an amplification band of the expected size after conventional PCR of all samples tested, indicating that expression of hsbp1 was switched-on in both control and PTHrP [67-86]-amide-treated cells. Subsequently, for semi-quantitative evaluation the cDNA preparations were submitted to SM-PCR as described. As shown in Fig. 1C, growth of 8701-BC cells in the presence of 1 nM PTHrP [67-86]-amide resulted in the upregulation of hsbp1 of approximately threefold with respect to control cells.

PTHrP [67-86]-amide upregulates hsp10, hsp90α, hsp90β, hsf1 and hsf2 in 8701-BC cells

The data obtained on the upregulation of hsbp1 prompted us to examine which was the expression pattern for other members of the stress protein family. In a second set of experiments we submitted cDNA samples from control and PTHrP-treated cells to conventional- and SM-PCR in the presence of primers specific for hsp10, -27, -60, -70, -90α, -90β, mthsp75, hsc70, grp78 and the two heat shock factors (hsf)-1 and -2. The panel in Fig. 2A shows that a positive signal was found for hsp10, -60, -90α and -90β, mthsp75, hsc70, grp78 and the two heat shock factors (hsf)-1 and -2. The panel in Fig. 2A shows that a positive signal was found for hsp10, -60, -90α and -90β, mthsp75, and for the two hsf; no amplification band was observed for hsp27 and -70, hsc70 and grp78, at least under the experimental conditions used. When cDNA preparations were submitted to SM-PCR to compare the expression levels of those stress protein genes selected on the basis of the previous results, we...
found that PTHrP [67-86]-amide was able to promote the upregulation of hsp10, hsp90α, hsp90β, hsf1 and hsf2 by 2.6-, 2.7-, 3.1, 7- and 4.7-fold, respectively (Fig. 2B). No statistically significant difference was found for the expression levels of hsp60 and mthsp75 between control and PTHrP [67-86]-amide-treated cells (not shown). Upregulation of hsf1, hsf2 and hsp90 in PTHrP-treated cells was also confirmed by western blot (Fig. 3).

Upregulation of hspb1 and hsp90 influences the expression of uPa and MMP-1 by 8701-BC cells

It is known that the human uPa and MMP-1 promoters contain binding sites for Ets transcription factors (Crawford and Matrisian, 1996; Yordy and Muise-Helmericks, 2000) (see also COMPEL database at http://compel.bionet.nsc.ru/compel/compel.html) and that hsf- and Ets-binding domains share a similar three-dimensional structure of winged helix-turn-helix DNA-binding sites (see PROFILE entry: QDOC50140 at http://www.isrec.isb-sib.ch), thus permitting Ets domain to be targeted by hsf1, which was proven to repress uPa expression (Chen et al., 1997). The results obtained with midregion PTHrP-treated cells indicated the upregulation of hspb1 and also of hsp90, which has been proven to associate with and sequester hsf1 thereby functioning as a powerful repressor for its activation (Zou et al., 1998; Knowlton and Sun, 2001).

In consideration of present and literature data, in a third set of assays we investigated whether upregulation of hspb1 and hsp90 could have some consequence on the levels of expression of uPa and MMP-1 genes, whose protein products are prominently involved in the acquisition of an invasive phenotype by breast cancer cells. As shown by conventional PCR analysis in Fig. 4, both uPa and MMP-1 transcripts were present in control and PTHrP-treated cells, although the intensity of the amplification signal for uPa was stronger in the preparation from treated cells, conversely that for MMP-1 being much fainter. Therefore, we examined the effect exerted on uPa and MMP-1 expression in PTHrP [67-86]-amide-treated 8701-BC cells.
by (1) the downregulation of hsbp1, (2) the functional inhibition of hsp90, and (3) the inactivation of hsfs. For (1), we designed a 15-mer ODN antisense to a theoretically accessible hybridisation site of the mRNA, according to RNAdraw software analysis and the suggestions of Ziegler et al. (Ziegler et al., 1999), and submitted cDNA preparations from mid-region PTHrP-treated cells cultured in the presence of either hsbp1-asODN or scrambled-asODN to SM-PCR for the evaluation of hsbp1 mRNA levels. For (2), we incubated PTHrP [67-86]-treated cells in the presence of geldanamycin, a benzoquinoid antibiotic from Streptomyces hygroscopicus which binds specifically and disrupts hsp90 function preventing its interaction with hsfs1 which, once freed, can be activated (Zou et al., 1998; Knowlton and Sun, 2001). For (3), we incubated untreated cells in the presence of the flavonoid quercetin, which is known to decrease the amount of free hsfs1 through downregulation of hsfs1 transcriptional activation and/or inhibition of hsfs1 activation (Hosokawa et al., 1992; Hansen et al., 1997), thus exerting a negative regulatory effect similar to that of hsbp1. Incubation with lipofectin-vehiculated hsbp1-asODN resulted in a 8.5-fold and 2-fold decrease of hsbp1 transcript amount with respect to parallel scrambled-asODN-treated controls cultured in PTHrP-containing medium, and to parallel untreated controls, respectively, suggesting a prominent RNase H-recruiting capacity owned by the selected hsbp1-asODN (not shown). The occurrence of asODN-induced cytotoxicity was also checked by MTS-tetrazolium-based assay after 24 hours incubation; no difference in the absorbance of produced formazan at λ=490 nm was found among untreated-, hsbp1-asODN-treated- and scrambled-asODN-treated cells (not shown), indicating that both asODNs, and also lipofectin treatment, were unable to affect the survival and proliferative behaviour of 8701-BC cells. Also geldanamycin- and quercetin-treated cell cultures displayed no changes in the amount of formazan accumulated after 24 hours’ incubation with respect to controls (not shown), indicating that these drugs did not exert any effect on cell viability and growth. Western blot analysis indicated that hsfs1, hsfs2 and hsp90 over-expression remained steady in all preparations from PTHrP-treated 8701-BC cells, irrespective of the supplement (Fig. 3).

Significantly, following addition of hsbp1-asODN we found a prominent downregulation (of approximately 6-fold) of uPa in midregion PTHrP-treated 8701 cells if compared to controls cultured in the presence of scrambled-asODN (Fig. 5, left panel); by contrast, an amplification band for MMP-1 was already detectable after 21 PCR cycles in cDNA samples from hsbp1-asODN treated cells, whereas no signal was observed in the control counterpart even if PCR cycles were increased up to 26 (Fig. 5, right panel A,B). Although to a lesser degree, a similar result was obtained when hsp90 was functionally blocked by geldanamycin: as shown in Fig. 5, left panel, following treatment, uPa was downregulated by approximately threefold, whereas a positive signal for MMP-1 was visible in cDNA samples from geldanamycin-treated cells from 44 cycles of amplification, being totally absent in control preparations (Fig. 5, right panel C,D). Interestingly, hsbp1-asODN
and geldanamycin treatment were also effective in increasing MMP-1 levels of expression in untreated 8701-BC cells by approximately 6- and 3-fold, respectively, whereas no significant variation was found for uPa expression level following treatments (not shown).

By contrast, analogously to PTHrP [67-86]-amide treatment, albeit to a lesser extent, incubation of 8701-BC cells with quercetin resulted in the upregulation of uPa (≥2-fold) and in the downregulation of MMP-1 (≥3-fold) (Fig. 6), thus providing further supporting data of the involvement of hsf unavailability on the modulation of uPa and MMP-1 expression.

Inhibition of hspb1 and hsp90 and inactivation of hsf1 influence the in vitro invasive behaviour of 8701-BC cells

On the basis of the expression data obtained, in a fourth set of assays we examined whether asODN-mediated downregulation of hspb1 and geldanamycin-dependent functional inactivation of hsp90 could restrain the ability of 8701-BC cells to penetrate an artificial basement membrane in Transwell chamber assay, as reported by Luparello et al. (Luparello et al., 1995); alternatively, the effect of quercetin on the invasive ability of 8701-BC cells seeded in plain RPMI 1640 medium was also tested. As shown in Fig. 7, hspb1-asODN- and geldanamycin treatments resulted in a prominent decrease of the amount of cells able to cross the matrigel-coated filters, the number of migrated cells/field being 7.35±0.5 and 1.5±0.4 (P < 0.001) for scrambled-asODN and hspb1-asODN-treated 8701-BC cells, and 6.5±0.6 and 2.2±0.4 (P = 0.002) for control and geldanamycin-treated 8701-BC cells (average±s.e.m.). Conversely, quercetin treatment resulted in a remarkable increase in cell invasive activity, the number of migrated cells/field being 1.9±0.39 and 7±0.73 (P < 0.001) for control and quercetin-treated 8701-BC cells (average±s.e.m.).

Discussion

Degradation of the basal lamina is one of the prerequisites for the malignant ingrowth of DIC at the primary site and it requires the expression of an enzymatic machinery able to lyse the complex network of cross-linked extracellular components, thereby facilitating the infiltration of metastatic tumor cells into the underlying stroma. During tumorigenesis, enzymes such as MMP-2 and -9 act on the collagen type IV component of the basement membrane, whereas interstitial collagenases and stromelysins play a subsequent role by breaking down the components of the interstitial connective tissue (for a review, see Duffy et al., 2000). It is generally acknowledged that the degradation of the extracellular matrix associated with metastatic spread is also mediated by several families of extracellular proteinases, including the serine proteinases, such as the uPa system and leukocyte elastases, and the cysteine proteinases, including tumor cell exterior-associated cathepsins B and L that promote tumor growth, invasion and
metastasis not only through lysis of extracellular matrices but also through endothelial cell growth-directed activities (for reviews, see Benaud et al., 1998; Krepela, 2001). Expression of extracellular proteinases, which in vivo can be found in both cancer and surrounding stromal cells, is known to be under the control of several cytokines and diffusible growth factors, such as EGF, PDGF, bFGF, TGFα or IGFs which were described as involved in the positive or negative regulation of MMPs and uPa (Aguirre Ghiso et al., 1999; Westermark and Kahari, 1999). Previous in vitro results obtained in our laboratory strongly suggested that a midregion fragment of PTHrP, i.e. PTHrP [67-86]-amide, is an invasion-promoting factor acting on cultured breast cancer cells, also indicating that matrigel penetration by PTHrP-stimulated cells was prominently impaired by inactivation of uPa. In the present paper we started a more detailed investigation into the molecular mechanisms underlying these functional observations; to the best of our knowledge, we present here the first evidence that the upregulation of some stress-related genes, most noticeably hsbp1 and hsp90s, whose protein product are known to interact and bind hsf, can intervene in the acquisition of an in vitro more invasive phenotype by breast cancer cells treated with PTHrP [67-86]-amide. The observed opposite regulation of hsf sequestering on MMP-1 and uPa expression could be explained on the basis of the reported effects of both hsf1 and Ets-1. In fact, it is known that hsf1 antagonizes the activating effects of the signal transducing protein Ras on uPa promoter (Chen et al., 1997); alternatively, hsf1, by targeting the Ets domain, could promote the upregulation of MMP-1, by analogy with the role of Ets-1 factor observed in some model systems (e.g. Naito et al., 2002; Ozaki et al., 2002).

Further studies will be required to determine a panel of midregion PTHrP-responsive genes in breast cancer cells and the related biological significance; however, present results allow us to make the following principal comments. First, the molecular data indicating a shift from a preferential MMP-1 to a preferential uPa-expressing phenotype obtained in the present work confirm previous postulate that over-secretion of uPa, rather than of other extracellular proteinases (i.e. other types of MMPs, trypsin-like enzymes, cysteine proteinases), was a primary condition for the increase of invasive activity triggered by PTHrP [67-86]-amide in vitro (Luparello et al., 1995; Luparello et al., 1997a). To our knowledge, a role played by PTHrP on the regulation of uPa activity was triggered by PTHrP [67-86]-amide, is an invasion-promoting factor acting on cultured breast cancer cells, also indicating that matrigel penetration by PTHrP-stimulated cells was prominently impaired by inactivation of uPa. In the present paper we started a more detailed investigation into the molecular mechanisms underlying these functional observations; to the best of our knowledge, we present here the first evidence that the upregulation of some stress-related genes, most noticeably hsbp1 and hsp90s, whose protein product are known to interact and bind hsf, can intervene in the acquisition of an in vitro more invasive phenotype by breast cancer cells treated with PTHrP [67-86]-amide. The observed opposite regulation of hsf sequestering on MMP-1 and uPa expression could be explained on the basis of the reported effects of both hsf1 and Ets-1. In fact, it is known that hsf1 antagonizes the activating effects of the signal transducing protein Ras on uPa promoter (Chen et al., 1997); alternatively, hsf1, by targeting the Ets domain, could promote the upregulation of MMP-1, by analogy with the role of Ets-1 factor observed in some model systems (e.g. Naito et al., 2002; Ozaki et al., 2002).

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Second, our investigation further supports the concept that breast cell differentiation state may be controlled by forms of PTHrP beyond those that have been studied most extensively to date, i.e. N-terminal and full-length PTHrP (e.g. Dunbar and Wysolmerski, 1999; Cataisson et al., 2000; Guise et al., 2002). Interestingly, PTHrP [67-86]-amide, which was also shown to be biologically active on placental transport of Ca2+/Mg2+ and on Ca2+-metabolism of squamous carcinoma cells (Care et al., 1990; Kovacs et al., 1996; Orloff et al., 1996) seems not to be a physiological midregion cleavage product of PTHrP, which according to Wu et al. (Wu et al., 1996) spans from aminoacid 38 to 94. We have previously reported that PTHrP [38-94]-amide impairs 8701-BC cell invasion through matrigel, which is the opposite to what was found with PTHrP [67-86]-amide (Luparello et al., 2001). Based on our collective observation, it will be worth examining which is the biological implication of the extra aminoacid sequence of 38-94 versus 67-86 fragment, and whether combinations of midregion PTHrPs, as well as of other N- and C-terminal PTHrP forms, may play a role in key functional steps of breast cancer invasion in vivo, thereby altering the net effect exerted by PTHrP on cancer progression.

Third, the demonstration of an effect exerted on the invasive properties of a breast tumor cell line treated with a fragment of PTHrP adds a new example to the hitherto very limited group of biological roles attributed to hsbp1. In fact, the only available data in literature report the influence of altered levels of hsbp1 on the survival of Caenorhabditis elegans exposed to different stresses, both chemical and thermal (Satyal et al., 1998). A biophysical and biochemical characterization of human hsbp1 has come out only very recently (Tai et al., 2002).

There are some questions whose answers will require further studies. First, the degree of the specific contribution of either hsp90α or -β to the induction of cell invasiveness was not examined because of the absence of specific drugs inactivating only one isoform, and the difficulty we encountered in inhibiting hsp90α or -β expression by asODN was probably because of the relatively long life of the proteins (data not shown), as also reported by Zou et al. (Zou et al., 1998).

Second, the selective upregulation of both hsf s tested is intriguing. An over-expression of hsf1 has been reported in a metastatic prostate cancer cell line and in most prostate cancer specimens examined by Hoang et al. (Hoang et al., 2000); no supporting data are available in the literature on the possible explanation of the upregulation of hsf2, whose protein product is generally acknowledged to control development and differentiation-specific gene expression, rather than responding to stress stimuli, by regulating genes distinct from those controlled by hsf1 (Pirkkala et al., 2001). The data reported here indirectly suggest that the equilibrium between bound and unbound hsf1 in PTHrP [67-86]-amide-treated 8701-BC cells is shifted to the former situation. Thus, whether the increase in the expression level can be interpreted as a cell response to the possible deficit of free hsfs, being sequestered by over-abundant hsbp1 and hsp90s, remains to be determined.

In conclusion, the results presented here have twofold significance: (1) they contribute to the knowledge of the cell biology of the diverse non-N terminal forms of PTHrP, further supporting that midregion PTHrP can be enclosed in the list of those elements potentially affecting breast cancer progression; and (2) they identify two new key protagonists in the complex scenario of DIC cell invasiveness in vitro, i.e. hsbp1 and hsp90, which deserve further and more extensive studies as potential and attractive molecular targets for the control of the malignancy of breast (and possibly other) cancer histotypes.

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