IDENTIFICATION OF A POTENTIAL TUMOR DIFFERENTIATION FACTOR (TDF) RECEPTOR FROM STEROID-RESPONSIVE AND STEROID-RESISTANT BREAST CANCER CELLS

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Background: Tumor Differentiation Factor (TDF) is a newly identified pituitary protein with no known receptor.

Result: Heat Shock 70 kDa proteins are potential TDF receptor candidates.

Conclusion: TDF acts on breast cells through a novel pathway.

Significance: These data may help elucidate the role of TDF.

SUMMARY

Tumor differentiation factor (TDF) is a recently discovered protein, produced by the pituitary gland and secreted into the blood stream. TDF and TDF-P1, a 20 amino acid peptide selected from the open reading frame of TDF, induce differentiation in human breast and prostate cancer cells, but not in other cells. TDF protein has no identified site of action or receptor and its mechanism of action is unknown. Here, we used TDF-P1, to purify and identify potential TDF receptor (TDF-R) candidates from MCF7 steroid-responsive breast cancer cells and non-breast HeLa cancerous cells using affinity purification chromatography (AP), and mass spectrometry (MS). We identified four candidate proteins from the 70 kDa Heat Shock Protein (HSP70) family in MCF7 cells. Experiments in non-breast HeLa cancerous cells did not identify any TDF-R candidates. AP and MS experiments were validated by AP and Western blotting (WB). We additionally looked for TDF-R in steroid-resistant BT-549 cells, and human dermal fibroblasts (HDF-a) using AP and WB. TDF-P1 interacts with potential TDF-R candidates from MCF7 and BT-549 breast cells, but not from HeLa or HDF-a cells. Immunofluorescence (IF) experiments identified GRP78, a TDF-R candidate, at the cell surface of MCF7, BT-549 breast cells and HeLa cells, but not HDF-a cells. IF of other HSP70 proteins demonstrated labeling on all four cell types. These results point towards GRP78 and HSP70 proteins as strong TDF-R candidates and suggest that TDF interacts with its receptor, exclusively on breast cells, through a steroid-independent pathway.

TDF is a protein produced by the pituitary gland and secreted into the blood stream, with no identified receptor and no known mechanism of action. TDF and TDF-P1, a 20 amino acid peptide selected from the open reading frame of TDF, induce morphological and biochemical changes in vitro and in vivo that suggest that TDF is involved in the differentiation of human breast (HBCC) and prostate cancer cells (1,2). Specifically, TDF induces markers of differentiation such as the polarization and formation of cell junctions and basement membrane, and furthermore induces milk protein synthesis and the over-expression of E-cadherin (3-10). However, TDF has no known morphological differentiation effect on fibroblasts or on kidney, hepatoma, and leukemic lymphocytic cell lines (1,2). The differentiation
activity of TDF has not been reproduced by any of the known pituitary hormones or growth factors (1,2). TDF is secreted by the pituitary directly into the blood, suggesting that this protein has an endocrine role (1,2). However, TDF protein is very under-studied. It is not yet clear where this protein acts and to what receptor it binds. It is also not clear how TDF protein promotes cell differentiation.

MCF7 human breast cancer cells express the estrogen receptors and are responsive to steroid hormones, manifested through activation of transcription of some genes, leading to increased cell proliferation (11-14). MCF7 human breast cancer cells are also responsive to TDF protein in vitro and in vivo through induction of cell differentiation (1,2). Therefore, it is of interest to understand whether TDF protein induces differentiation of MCF7 breast cancer cells through a steroid-dependent or steroid-independent pathway. It is of additional interest as to whether the TDF pathway is similar to the estrogen pathway or is a novel pathway.

The first step in understanding the TDF pathway is through the isolation and characterization of the TDF-R. The standard procedure for isolation and characterization of most membrane-bound or intracellular receptors for hormones or growth factors is through AP and Edman sequencing or MS (15,16). Due to its higher accuracy, sensitivity, cost and speed, MS has become the method of choice for identifying and sequencing proteins (15,17-20). Validation of these findings is typically performed using AP, followed by WB using antibodies against TDF-R candidates identified by MS. If validation is positive, then the potential receptor (or receptors) warrant(s) further investigation.

Here, we used TDF-P1 to purify potential TDF-R candidates from MCF7 steroid-responsive breast cancer cells and non-breast HeLa cancerous cells using AP and MS. We used TDF-P1 because we reasoned that if TDF-P1 mimics the effect of full length TDF protein and induces cell differentiation, then TDF-P1 must interact with the receptor of full-length TDF and therefore, TDF-P1 could be used to purify the potential TDF receptor candidates. We further investigated the potential TDF-R in these two cell types and additionally in steroid resistant BT-549 cells (these cells do not express estrogen receptors) (21) and HDF-a by AP, WB, and IF. Our results suggest that TDF-R candidates are members of the HSP70 protein family, are present on breast and cancerous cells, but not other cells, and act through a novel steroid-independent pathway. The possibility that TDF-R is a multi-subunit, protein complex is also discussed.

Experimental Procedures

**Peptide synthesis and coupling to agarose beads.** TDF-P1 peptide, with the amino acid sequence NH2-RESQGTRVGQALSFLCKGTA-COOH was synthesized by standard peptide synthesis (Creative Biolabs) and its correct sequence was confirmed by MS (data not shown). The TDF-P1 peptide was then coupled to CNBr activated beads (Sigma) according to manufacturer instructions.

**Cell culture & cell lysis.** All cell lines were grown in RPMI 1650 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin (P/S; all from GIBCO, Invitrogen Corporation, Carlsbad, CA), and were grown to confluence under standard cell culture conditions (5% CO2 & 37°C). The cells were then washed with cold PBS pH 7.4 and then lysed using lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 1% (v/v) Triton-X-100, and SIGMAFAST™ Protease Inhibitor Tablets (Sigma-Aldrich 1 tablet/100 mL buffer)]. The cell lysate was pooled to have uniform concentration of proteins and then split into 1 mL aliquots in Eppendorf tubes. These tubes were then incubated on ice for 30 minutes and centrifuged for 20 minutes at 14,000 rpm at 4°C in an Eppendorf centrifuge (Epifuge). The pellet contained DNA and unsolubilized material and was discarded. The supernatant was collected and combined and then stored at -80°C for further experiments. A control experiment in which the...
beads alone were used as bait for the AP experiments, was also performed. The eluates from the control AP experiments were also analyzed by MS and WB.

**Immunoaffinity precipitation (IAP).** Recombinant human GRP78 protein was obtained from ProSpec-Tany TechnoGene Ltd. (Rehovolt, Israel). GRP78 protein at a concentration of 1\(\mu\)g/100\(\mu\)l was incubated with TDF-P1 (at a concentration of 10\(\mu\)g/100\(\mu\)l) in lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 1% (v/v) Triton-X-100, and SIGMAFAST\textsuperscript{TM} Protease Inhibitor Tablets (Sigma-Aldrich, St. Louis, MO; 1 tablet/100 mL buffer)] for 2 hours at 4°C. The mixture of GRP78 protein and TDF-P1 was then incubated for 2 hours with 1\(\mu\)g of mouse monoclonal anti-GRP78 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G UltraLink Resin (Thermo Scientific, Waltham, MA). Samples were then centrifuged for 30 s at 3,000 rpm and the supernatant was saved for analysis. Pellet was washed 3 times with ice cold lysis buffer and boiled for 10 minutes in Laemmli sample buffer. A negative control with protein A/G and no antibodies was processed in the same manner. All samples were loaded on 16% Tris-HCl gels and separated by SDS-PAGE. Because the molecular mass of the TDF-P1 is small (about 2 kDa), the gels were stopped before the front electrophoresis ran out of the gel. The gels were then electroblotted, incubated with relevant primary antibody, produced in a different host than the antibodies used for immunoprecipitation – rat monoclonal GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G UltraLink Resin (Thermo Scientific, Waltham, MA). Samples were then centrifuged for 30 s at 3,000 rpm and the supernatant was saved for analysis. Pellet was washed 3 times with ice cold lysis buffer and boiled for 10 minutes in Laemmli sample buffer. A negative control with protein A/G and no antibodies was processed in the same manner. All samples were loaded on 16% Tris-HCl gels and separated by SDS-PAGE. Because the molecular mass of the TDF-P1 is small (about 2 kDa), the gels were stopped before the front electrophoresis ran out of the gel. The gels were then electroblotted, incubated with relevant primary antibody, produced in a different host than the antibodies used for immunoprecipitation – rat monoclonal GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal TDF generated against TDF-P1 (Creative Biolabs, Shirley, NY). Secondary antibodies were HRP-conjugated: goat-anti-rat IgG-HRP and goat-anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz CA). The signal was visualized by an enhanced chemiluminiscent (ECL) reaction kit (Pierce, Rockford, IL).

**SDS-PAGE and WB.** The eluates from obtained from AP experiments were solubilized for 5 minutes in Laemmli sample buffer/95°C, loaded on 8% Tris-HCl gels and separated by SDS-PAGE. In some experiments, the APs were first concentrated five times on a low Mw Amicon concentrator (Millipore, Bedford, MA). The gels were then stained by Coomassie or electroblotted on Biotrace\textsuperscript{*} NT nitrocellulose membrane (Pall\textsuperscript{*} Life Sciences, Port Washington, NY), incubated with different commercial primary GRP78, HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA) and HPR conjugated secondary antibodies: goat-anti-rat IgG-HRP, goat-anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz CA) and then visualized by an enhanced chemiluminiscent (ECL) reaction kit (Pierce, Rockford, IL). The Coomassie stained gels were the starting material for MS-based analysis.

**Protein digestion and peptide extraction.** Proteins that were separated by SDS-PAGE and stained by Coomassie dye were cut in 4-6 gel pieces and washed in high purity, high performance liquid chromatography HPLC grade water, cut into small pieces and destained by incubating in 50 mM ammonium bicarbonate, 50 mM ammonium bicarbonate/50% acetonitrile, and 100% acetonitrile under moderate shaking, followed by drying in a speed-vac concentrator (22). The gel bands were then rehydrated with 50 mM ammonium bicarbonate. The procedure was repeated twice. The gel bands were then rehydrated in 50 mM ammonium bicarbonate containing 10 mM DTT and incubated at 56°C for 45 minutes. The DTT solution was then replaced by 50 mM ammonium bicarbonate containing 100 mM Iodoacetamide for 45 minutes in the dark, with occasional vortexing. The gel pieces were then re-incubated in 50 mM ammonium bicarbonate/50% acetonitrile, and 100% acetonitrile under moderate shaking, followed by drying in a speed-vac concentrator. The dry gel pieces were then rehydrated using 50 mM ammonium bicarbonate containing 10 ng/\(\mu\)L trypsin and incubated overnight at 37°C under low shaking. The resulting peptides were extracted twice with 5% formic acid/50 mM ammonium bicarbonate/50% acetonitrile and once with 100% acetonitrile under moderate shaking. The peptide mixture was then dried in a speed-vac, solubilized in 20 \(\mu\)L of 0.1% formic acid/2% acetonitrile.

**MS and protein identification.** The resulting peptide mixture was analyzed by reverse phase liquid chromatography (LC) and MS (LC-MS/MS) using an Alliance 2695 (Waters Corp, Milford, MA) coupled to a Q-Tof Micro MS (Micromass/Waters, Milford, MA). The peptides were loaded onto an XBridge\textsuperscript{TM} C18 3.5 \(\mu\)m, 2.1 x 100 mm column (Waters Corporation, Milford,
MA) and eluted over a 60 minutes gradient of 2-100% acetonitrile in 0.1% formic acid at a flow rate of 200 μL/min. MS data acquisition involved survey MS scans and automatic data-dependent MS/MS of 2+, 3+ or 4+ ions. The MS/MS was triggered when the MS signal intensity exceeded 10 counts/second. In survey MS scans, the three most intense peaks were selected for collision-induced dissociation (CID) and fragmented until the total MS/MS ion counts reached 5,000 or for up to 6 seconds each. Additional experiments were performed using a nanoLC-MS/MS that contained a Micromass-QTOF hybrid mass spectrometer (Waters Corporation, Milford, MA) with a nanoelectrospray source. A fused silica tip mounting adaptor, fitted with a 75-μm (inner diameter) fused silica tip (New Objective), was connected through a 50-μm (inner diameter) fused silica tubing to the liquid chromatography (LC) detector outlet. An LC Packings system (Dionex Corp., Sunnyvale, CA), equipped with an Ultimate micro pump and solvent organizer and a Switchos loading pump and Famos autosampler, was used for LC-MS. Separation was carried out on a 75-μm x 15-cm column (LC Packings C18 PepMap; 5-μl injection volume) at a flow rate of 200 nl/min, using a gradient of 2–80% ACN in 0.1% FA. The mass spectrometer was operated in the data-dependent mode and automatically switched between MS and MS/MS. In survey MS scans, the seven most intense peaks were selected for collision-induced dissociation (CID) and fragmented. Full description of the nanoLC-MS/MS analysis and data processing is described elsewhere (23-25). The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software with the following parameters: background subtraction of polynomial order 5 adaptive with a threshold of 35%, two smoothings with a window of three channels in Savitzky-Golay mode and centroid calculation of top 80% of peaks based on a minimum peak width of 4 channels at half height. The resulting pkl files were submitted for database search and protein identification to the public Mascot database search (www.matrixscience.com, Matrix Science, London, UK) using the following parameters: human databases from NCBI and SwissProt, parent mass error of 1.2 Da, product ion error of 0.6 Da, enzyme used: trypsin, one missed cleavage, and carbamidomethyl-Cysteine as fixed modifications and Methionine oxidized as variable modification. To identify the false negative results, we varied our database search parameters and compared the results with each other. To eliminate false positive results, we manually checked the MS/MS spectra that led to identification of a protein. The pkl files were also searched against in-house PLGS database (www.waters.com) using searching parameters similar to the ones used for Mascot search. The Mascot and PLGS database search provided a list of proteins for each gel band. Only proteins identified by two or more peptides and with a Mascot score >50 were considered. For the proteins identified by a mascot score lower than 50, the MS/MS spectra of these peptides were manually inspected to confirm the identity of the peptide.

**IF.** Cells were grown for 24 hours on coverslides in 6-well plates and washed with PBS 3 times before staining using 3 µM CM-Dil (Invitrogen Corporation, Carlsbad, CA) for 5 minutes at 37°C followed by 15 minutes at 4°C. Afterwards, cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature (RT). They were then incubated in 0.1% Triton 50 mM NH_4Cl/ PBS for 5 minutes at RT. Cells were blocked for 60 min at RT in blocking buffer (10% normal donkey serum in Tris-buffered saline pH 7.4 – TBS) and then incubated with relevant primary antibody; GRP78, HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for 60 minutes at RT, washed with TBS and followed by secondary antibody, Alexa Fluor 488-conjugated 1:500 (Invitrogen, Carlsbad, CA) for 30 minutes at RT in the dark. Cells were washed with TBS, nuclei were counterstained with 0.5µM DAPI (Sigma) for 4 minutes at RT and coverslides were mounted using VectaShield HardSet mounting medium. All images were obtained using a Nikon Eclipse TE200 inverted microscope and were processed using IPLab 4.0.

**Peptide Docking to HSP proteins.** The tentative peptide (TDF-P1) docking sites were computationally predicted using pdb crystal structures 1YUW (26), 3LDN (27), 3N8E (28), and 2E88 (29) as receptors. 1YUW is a bovine heat shock 70 kda protein 8, composed of nucleotide binding domain (NBD) and the substrate binding domain (SBD) β. 1YUW shares
68%, 88% and 55% sequence identity with GRP78, HSP1 and HSPA9, respectively (30). 3LDN corresponds to the human GRP78 ATPase domain (apo form). 3N8E is a substrate binding domain of human heat shock protein. It is a heat shock 70kda Protein 9. 2E88 is the apo-form of the ATPase domain of human heat shock 70kda protein 1.

Following previously reported framework of "blind docking," we have used the whole protein chain as a model receptor to identify the potential peptide binding regions (31). The docking operation was carried out using GRAMM-X Protein-Protein Docking Web Server v.1.2.0. (32,33). The simulation generated several protein receptor/peptide conformations with an output in the form of a pdb file. Graphic views of the proposed docked model simulations were generated using Discovery Studio Visualizer 3.1. To substantiate these results further, “Patch dock” and “Fire dock” simulation servers were used to develop another set of models. To do this, preliminary models were developed using “Patch dock” and refined again by using “Fire dock” (34-37).

RESULTS

Protein Isolation and identification of TDF-R candidates by AP and MS. Isolation of the TDF-R was performed by AP, followed by MS. We synthesized TDF-P1 peptide, coupled the peptide to agarose beads, and incubated the peptide beads with the cell lysate from estrogen-responsive MCF7 human breast cancer cells. After incubation, we washed the beads, eluted the TDF-R candidates and separated them by SDS-PAGE. The gel bands were excised and digested with trypsin and the peptide mixture was then analyzed by LC-MS/MS. We analyzed all gel bands from each lane.

Isolation and identification of TDF-R candidates from steroid-responsive MCF7 breast cancer cells. In LC-MS/MS experiments performed with AP eluate isolated from estrogen-responsive MCF7 cells, we identified four proteins with high confidence: Glucose regulated protein (GRP78, gi386758), heat shock 70kDa protein 8 isoform 1 (HSP8, gi5729877), heat shock 70kDa protein 1 (HSP1, gi4529893), and Heat shock 70kDa protein 9 (HSPA9, gi12653415). Glucose regulated protein (GRP78, gi386758) is a 78 kDa protein and a member of the HSP family, also named Heat shock 70 kDa protein 5 or immunoglobulin heavy chain-binding protein (BiP). The other three proteins identified in our experiments, HSP8 (gi5729877), as HSP1 (gi4529893), and HSPA9 (gi12653415), were all part of the HSP70 kDa protein family. Example of Total ion current (TIC), MS and MS/MS of peptides identified in our LC-MS/MS experiments, which were part of GRP78, HSP1, HSP8 and HSPA9 are shown in Figure 1 (GRP78), Figure 2 (HSP1), Figure 3 (HSP8) and Figure 4 (HSPA9).

Additional structural proteins such as actin, keratin, cytokeratin and tubulin were also identified. In control experiments, where we used only agarose beads without TDF-P1 peptide, we identified cytokeratins (8 and 18), actin and tubulin, suggesting that these proteins are false positive identifications in our experiments. However, in these control experiments we did not identify any protein that was part of the HSP family, suggesting that these proteins are true TDF-R candidates. Examples of TIC, MS and MS/MS of peptides that were part of cytokeratin 8 and 18 are shown in Supplemental Figures 1 & 2. A summary of the peptides and proteins identified in our AP and MS experiments using MCF7 cells is shown in Table 1. Taken together, these experiments suggest that GRP78, HSP1, HSP8 and HSPA9 proteins are potential TDF-R candidates.

Isolation and identification of TDF-R candidates from non-breast HeLa cancerous cells. To investigate whether the TDF-R candidates can also be purified by AP and identified by MS, we analyzed, in addition to MCF7 cells, non-breast HeLa cancerous cells. In our AP and MS experiments using TDF-P1 peptide to purify the TDF-R candidates from HeLa cancer cells, we did not identify any protein. In control experiments, in which we used only agarose beads for AP from the lysate of HeLa cells, followed by MS, we did not identify any protein. These experiments suggest that non-breast HeLa cells either do not have TDF-R at the cell surface or do not have the receptor at all, or TDF-P1 peptide does not interact with the potential TDF-R candidates.

Validation of AP and MS experiments. To validate our experiments, we performed a similar AP experiment followed by WB using antibodies
against GRP78 and against HSP70 protein. While anti-GRP78 antibodies were specific against human GRP78 protein, the anti-HSP70 protein antibodies were made against whole HSP70 protein and part of its amino acid sequence was also common to the amino acid sequences of HSPA9, HSP8, and HSP1 (all of them part of HSP70 protein family). Due to the small differences in the Mw, we could not differentiate between HSP8 (70 kDa), HSP1 (70 kDa), and HSPA9 (72 kDa) proteins, and, since they were already identified by MS, we considered that the anti-HSP70 antibodies identified all HSP8, HSP1 and HSPA9 proteins.

**HSP70 proteins are potential TDF-Rs in steroid-responsive MCF7 cells.** When we analyzed the MCF7 cells by AP and WB using anti-GRP78 and anti-HSP70 antibodies, we could observe an immune reaction at around 73 kDa with anti-HSP70 antibodies and around 78 kDa with anti-GRP78 antibodies (Figure 5A). In an additional WB experiment, when a more concentrated eluate was used, a stronger reaction was observed (Supplemental Figure 3). In control experiments of AP using beads alone, followed by WB with anti-GRP78 and anti-HSP70 antibodies, no immune reaction was observed (data not shown). Therefore, the interaction of TDF-P1 peptide with GRP78 and HSP70 proteins, previously found by AP and MS are true interactions and GRP78 and HSP70 proteins are true potential TDF-R candidates.

**HSP70 proteins are potential TDF-Rs in both steroid-responsive MCF7 cells and steroid-resistant BT-549 cells.** To investigate whether TDF-P1 interacts with GRP78 and HSP70 proteins in steroid-resistant human breast cancer cells (BT-549 cell line), we performed an AP and WB using anti-GRP78 and anti-HSP70 antibodies. In BT-549 cells, we observed the same immune reaction as previously observed in MCF7 cells with antibodies against both GRP78 and HSP70 (Figure 5B). In an additional WB experiment, when a more concentrated eluate was used, a stronger reaction was observed (data not shown). These results suggest that that GRP78 and/or HSP70 proteins are receptors for TDF protein in both MCF7 steroid responsive and BT-549 steroid resistant cells. These results also suggest that TDF protein activates, through GRP78 and/or HSP70 proteins, a pathway that is different from the steroid pathway.

**HSP70 proteins are potential TDF-Rs in breast cancer cells, but not in HeLa cancer cells.** To further investigate whether TDF-P1 interacts with GRP78 and HSP70 proteins in other, non-breast, cells, we chose HeLa cancer cells for further investigation. These cells are not steroid regulated cells and do not contain steroid receptors, and therefore we can eliminate any possible interference of the TDF-R pathway with the steroid receptor pathway. We performed an AP experiment from HeLa cell lysate using TDF-P1 as bait, followed by WB using the same anti-GRP78 and anti-HSP70 antibodies. As observed in Figure 5C, no reaction was observed in the AP and WB experiments when anti-GRP78 or anti-HSP70 antibodies were used. In an additional WB experiment, when a more concentrated eluate was used, again, no reaction was observed (Supplemental Figure 3). These results suggest that GRP78 and HSP70 proteins are potential TDF-R candidates in breast cancer cells, but not in other cancer cells (HeLa).

**HSP70 proteins are potential TDF-Rs in breast cancer cells, but not in non-breast, normal HDF-a.** To investigate whether GRP78 and/or HSP70 proteins are TDF-R candidates in non-breast, non-cancer cells, we investigated HDF-a using AP and WB experiments that utilized anti-GRP78 or anti-HSP70 antibodies (Figure 5D). As observed, no reaction was observed in the AP and WB experiments when anti-GRP78 or anti-HSP70 antibodies were used. We would, however, like to mention that HDF-a cells grew slower and the cell lysate that was accumulated and used for AP and WB experiments was about eight times lower compared with the other cells. However, in an additional WB experiment, when a more concentrated eluate was used, no reaction was observed (data not shown). Therefore, GRP78 and HSP70 proteins are potential TDF-R candidates in breast cells, but not in other cells (HeLa and HDF-a). These data suggests that the TDF protein has its own pathway that is independent of the steroid pathway and is restricted to breast cells.

**Characterization of expression of GRP78 protein in steroid-responsive MCF7 cells, steroid-resistant BT-549 cells, non breast cancerous HeLa cells and normal HDF-a.** Identification of GRP78 and HSP70 family members of proteins as
potential TDF-R candidates in breast cancer cells warranted further IF investigation. Therefore, to investigate whether HSP70 proteins that were identified as potential TDF-R candidates are expressed on the cell surface of breast cells, but not on HeLa cancerous cells or on HDF-a fibroblasts, we analyzed these cell lines by IF. We used antibodies against GRP78 and HSP70 proteins to track expression of both proteins in all investigated cell lines. The outcome of this experiment is presented in Figures 6-7. As expected, GRP78 protein was expressed in the plasma membrane fractions of both steroid-responsive MCF7 and steroid-resistant BT-549 cells (Figure 6). Staining can be observed not only in the cytosol but also in the membrane of the cells. Membrane was also stained using selective membrane tracker CM-Dil and it can be clearly seen that GRP78 is detected outside the cells. This experiment is consistent with the AP and MS experiments and with AP and WB experiments, and further suggests a possible role of GRP78 as a receptor for TDF protein. A similar expression pattern of GRP78 protein was observed when non-breast HeLa cancer cells were used (Figure 6). However, when normal HDF-a cells were used, no staining outside the cells was observed (Figure 6). Both HeLa and HDF-a exhibited much weaker GRP78 membrane staining, further confirming that GRP78 might be a TDF-R candidate selective only for breast cell lines.

**Characterization of expression of HSP70 proteins in steroid-responsive MCF7 cells, steroid-resistant BT-549 cells, non breast cancerous HeLa cells and normal HDF-a.** When IF experiments using anti-HSP70 antibodies were performed, the staining was similar in all four cell lines studied (MCF7, BT-549 breast cancer cell lines, HeLa cancer cells, and HDF-a cells, Figure 7). Staining was observed both in the cytosol and in the membrane of the cells. Membrane was also stained using selective membrane tracker CM-Dil and it can be clearly seen that HRP is detected outside the membrane of all cells studied. Taken together, the IF experiments suggest that, if GRP78 protein is a true potential TDF-R candidate. These results corroborated with AP and MS and with AP and WB experiments, suggest that GRP78 protein is the main receptor of TDF protein and HSP70 proteins are contaminants co-purified together with GRP78 protein.

Alternatively, TDF-R may have GRP78 as a main receptor and HSP70 proteins as co-receptors.

**Investigation of the interaction between TDF-P1 and the HSP70 family of proteins.** To further investigate whether TDF-P1 and HSP70 proteins interact with each other and to further confirm the AP and MS experiments (Figures 1-4) and the AP and WB experiments (Figure 5), we used two additional approaches: 1) direct infusion followed by electrospray ionization mass spectrometry (ESI-MS) and 2) immunoaffinity purification (IAP) of GRP78/BiP preincubated with TDF-P1, followed by WB using anti-GRP78 and anti-TDF antibodies (IAP & WB). Using the first approach, we monitored the levels of TDF-P1 in the ESI-MS, either alone or when the TDF-P1 peptide was pre-incubated with Myoglobin, bovine serum albumin (BSA) or recombinant GRP78/BiP. We reasoned that if TDF-P1 interacts with one of these proteins, then we should observe the disappearance of TDF-P1 from the ESI-MS spectrum, mostly due to its interaction with one of the three proteins analyzed. The outcome of this experiment is shown in Supplemental Figure 4. TDF-P1 was observed mostly as a (2+) double-charged peak with m/z of 1106.59 and as a triple-charged (3+) peak of m/z of 738.09 (Supplemental Figure 4A), but variation in the m/z of the measured triple-charged peak was also observed (e.g. m/z of 738.075 & 738.1144; Supplemental Figures 4B & C). When TDF-P1 was pre-incubated with Myoglobin or BSA, a large amount of TDF-P1 was observed in its monomeric form and its relative intensity in these two spectra was not different from its intensity of the TDF-P1 peptide measured alone, suggesting that TDF-P1 does not interact with Myoglobin or with BSA. However, when TDF-P1 was pre-incubated with recombinant GRP78/BiP, almost no TDF-P1 could be identified in its monomeric form, suggesting that TDF-P1 interacts naturally with GRP78/BiP. We also investigated the interaction of TDF-P1 with these three proteins by monitoring the shift in m/z of these proteins before and after incubation with TDF-P1. Although we detected no mass shift between Myoglobin and Myoglobin pre-incubated with TDF-P1 or between BSA and BSA pre-incubated with TDF-P1, the results using GRP78/BiP and GRP78/BiP pre-incubated with TDF-P1 were inconclusive (data not shown).
To further investigate whether TDF-P1 interacts with the members of the HSP70 family of proteins, we pre-incubated GRP78 with TDF-P1 and then purified them by IAP using anti-GRP78 antibodies. The IAP eluate was then tested by WB using antibodies against TDF-P1 and GRP78. We reasoned that WB of the IAP using antibodies against TDF-P1 and GRP78 should demonstrate a direct interaction between GRP78 and TDF-P1. The outcome of the experiment is shown in Figure 8. When the GRP78-TDF-P1 mixture was analyzed by anti-GRP78 or anti-TDF-P1 antibodies, both the protein and the peptide were detected (Figure 8 lane 1). When the IAP was performed using anti-GRP78 antibodies, no protein was detected in the IAP flow-through (Figure 8 lane 2). Protein A/G-bound beads used as a negative control also showed no reaction (Figure 8 lane 3). However, when the IAP eluate was tested by WB, it reacted with both GRP78 and TDF-P1 antibodies (Figure 8 lane 4). Taken together, these data suggest that TDF-P1 indeed interacts naturally with GRP78/BiP, but not with other proteins such as Myoglobin or BSA, consistent with our previous finding.

Investigation of interaction between TDF-P1 and HSP70 family of proteins using structural biology (peptide docking). We also investigated whether TDF-P1 interacts with native GRP78 and with members of HSP70 family of proteins using structural biology. Specifically, we investigated docking of TDF-P1 on binding sites of GRP78 or HSP70 proteins, using the available crystal structure of these proteins. The outcome of this experiment is presented in Figure 9. The TDF-P1 peptide binding pockets were predicted using pdb structures 1YUW (Figure 9A and B), 3LDN (Figure 9C-E), 3N8E (Figure 9F-H) and 2E88 (Figure 9I and J).

The first 10 most optimal scoring structures that resulted from the “GRAMM-X” simulation were considered for detailed scrutiny. The frequencies with which the locations of the docking pockets appeared among these models were used as the primary criteria for determining the most probable structure. The order of sequencing of the results was also used as a second condition for selection. Based on these considerations, we selected two docked models from this list. Figure 9A and 9B shows these two docked models in the case of 1YUW, indicating the most probable (tentative) binding pockets. Six of the top ten simulations had almost the same docking site as Figure 9A. Two of the ten simulations had almost the same binding pocket as Figure 9B. The results using “Patch dock” and “Fire dock” simulation servers were almost identical to those shown in Figure 9A and 9B, with the relative frequencies of the different probable binding pockets slightly differing between the different sets of runs.

Figure 9C-E, describes tentative models of TDF-P1 binding pockets in 3LDN (Chain A). After carrying out the docking operation using “GRAMM-X”, four of the ten simulations had almost the same docking region as that shown in Figure 8C. In three of these ten simulations, the TDF-P1 peptide docked at the binding site identified in Figure 9D and two of the results had almost the same configuration as the one shown in Figure 9E. Results similar to those of Figure 9C and 9D were also obtained using “Patch dock” and “Fire dock”.

The TDF-P1 binding pocket was predicted using pdb structure 3N8E (chain A). Figure 9F-H describes the tentative models of peptide binding sites in SBD of human heat shock 70kda Protein 9. The docking operation was carried out using “GRAMM-X”. Six of the ten simulations had nearly the same binding pockets as Figure 9F. One of the ten simulations essentially mimicked the binding configuration of Figure 9G, and in two cases, the docking occurred approximately in the same region as that shown in Figure 9H. Results very similar to that of Figure 9G were also obtained using “Patch dock” and “Fire dock”. In the last two cases, however, only a single binding location, similar to the one mentioned above was observed.

Figure 9I and J describes tentative models of TDF-P1 binding regions in the ATPase domain of human heat shock 70kda protein 1 (PDB ID 2E88). The docking operation was carried out using “GRAMM-X”. One of the ten simulations yielded the same binding region as that shown in Figure 9I. In six of these ten simulations, the peptide docked in the binding site identified in Figure 9I. Additional results similar to those of Figure 9I were also obtained using “Patch dock” and “Fire dock”. In the latter case, however, the configuration considered in Figure 9I remained excluded. It is worth noting that the simulations
reported here did not account for possible structural changes of the receptor protein upon peptide binding.

**DISCUSSION**

TDF is a relatively new protein secreted by the pituitary into the blood stream, with no definitive function and no known receptor. It is an under-investigated protein, yet to be universally recognized as a new hormone. The first step that we took in unveiling the function of TDF was to isolate its receptor. In our experiments using AP and MS, we identified four proteins that are potential TDF-R candidates: GRP78, HSP8, HSP1 and HSPA9. All these proteins are all members of the HSP70 family of proteins and are all involved in the folding and assembly of proteins in the endoplasmic reticulum (ER), but they may also be identified in the cytosol or cell membrane (38-48).

Function of HSPs. HSPs are highly expressed in cancerous cells and are essential to the survival of these cells (49-58) and therefore, HSP inhibitors show promise as anticancer agents (27,59-66). HSPs (Hsp70 and Hsp90) have been associated with both estrogen and androgen receptors (67-71). HSPs have a role in cell proliferation. For example, inhibition of HSP90 led to the dysregulation of HPS70 and inhibition of cell proliferation (61). HSPs also have a role in apoptosis and cell differentiation, especially HSP70 and HSP90. These two proteins interact with apoptotic proteins and block the apoptotic pathways, thus promoting cell differentiation (61). HSPs may even determine whether cells undergo apoptosis or differentiation (44). Recently it was demonstrated that GRP78 forms a cell surface complex with Cripto, an oncoprotein that signals via MAPK/ERK, PI3K/Akt and Smad2/3 pathways, and mediates signaling in human tumor, mammary epithelial and embryonic stem cells (43). Active Cripto from Cripto-GRP78 complex promotes cellular proliferation, decrease of cell adhesion and down-regulation of E-Cadherin. However, Cripto alone is not able to signal and promote the above-mentioned cellular events. Therefore, GRP78, when in complex with Cripto, is an oncogene (43). When it is not in a protein complex with Cripto, it may promote cell differentiation (44).

In our AP experiments using estrogen-responsive MCF7 cells, we expected to identify estrogen receptors, but instead we identified HSP70 proteins. Therefore, TDF may have a natural receptor that is different from estrogen receptors and which promotes differentiation of steroid-responsive cells by a non-steroid signal transduction pathway. We wanted to investigate whether TDF protein has HSP70 proteins as TDF-Rs only on MCF7 estrogen-responsive breast cancer cells or in both estrogen-responsive and -resistant breast cancer cells. We therefore investigated BT-549 estrogen-resistant breast cancer cells in AP and WB experiments using antibodies against GRP78 and HSP70. In BT-549 cells, we identified the same HSP70 proteins as receptors for TDF protein as we had previously found in MCF7 cells, suggesting that the TDF acts both on steroid-responsive and steroid-resistant breast cancer cells through the same TDF-R, but through a pathway independent of estrogen receptors and independent of the estrogen pathway.

TDF-R was identified in MCF7 cells and BT-549 cells, both of which are breast cancer cells. This indicates that HSP70 proteins may act as TDF-R specifically in in cancerous cells, in breast cells or in both types of cells. To investigate whether other cancerous, non-breast cells have the same TDF-R candidates we analyzed non-breast HeLa cancerous cells. We found that the AP did not identify any HSP70 proteins in either AP and MS or AP and WB. Therefore, it seems that TDF-R is linked somehow to breast cells specifically and not globally to cancer cells. To further investigate whether TDF has a TDF-R (or interacts with it) only in breast cells or in any (non-cancerous) cells, we investigated HDF-a fibroblasts by AP and WB. As in HeLa cells, we did not see any reaction, again suggesting that TDF-R is somehow linked to breast cells and is not present (or it does not interact with its ligand) in normal or cancerous cells.

It is possible that we identified GRP78 as a potential TDF-R candidate, since GRP78, when present at the cell surface, has many roles, acting as a hub for signaling to the nucleus (42,50,66,72-79). We should also remember that TDF-P1 peptide produces a differentiation effect on breast and prostate cancer cells (2). However, it does not make sense that GRP78 and the other HSP70
proteins are specific only to breast cells and not to
cancer or non-cancer cells, unless 1) a specific
number of receptors act as co-receptors for TDF
and 2) only when all co-receptors are in the same
place and at the same time as a protein complex
receptor, TDF binds to its receptor and promotes
cell differentiation.

To further investigate whether the HSP70
proteins are localized at the cell membrane, we
investigated MCF7, BT-549, HeLa and HDF-a cell
lines by IF using anti-GRP78 and anti-HSP70
antibodies. While GRP78 was identified at the cell
membrane of MCF7, BT-549 and HeLa cells, the
other HSP70 proteins were identified in all cell
lines investigated. It should also be kept in mind
that GRP78 and other HSP70 proteins have been
identified at the cell surface of all cancer cell lines
investigated in the current study and in other
studies (49-58), but they were not identified by AP
and MS or AP and WB in any non-breast cancer
cell line that we investigated. Therefore,
comparison of AP and MS with AP and WB and
further using IF suggest that TDF-R is restricted to
breast cells. These data also suggest that GRP78 is
the main receptor for TDF-R and the other HSP70
proteins are either co-receptors for TDF protein or
isolation artifacts.

In conclusion, we used AP and MS
experiments to investigate different cell lysates to
identify TDF-R candidates and AP and WB, ESI-
MS, IAP &WB and structural biology experiments
to further validate our findings. We found that all
TDF-R candidates identified are members of the
HSP70 family, (possibly as a protein complex),
and were detected only in breast cancer cells, but
not in non-breast HeLa cancerous or normal non-
breast HDF-a cells. The HSP70 proteins were
found in both MCF7 steroid responsive cells and
BT-549 steroid resistant cells. This suggests that
TDF protein acts in both cell types through a
steroid-independent receptor and activates a TDF
pathway that is specific to breast cells. All four
HSP70 proteins are being further investigated as
potential TDF-R candidates.

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**FOOTNOTES**

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The abbreviations used are: TDF, Tumor differentiation factor; TDF-R, TDF-Receptor; HBCC, human breast cancer cells; MCF7, steroid-responsive breast cancer cells; BT-549, steroid-resistant breast cancer cells; HeLa, cancer cells; HDF-a, human dermal fibroblasts; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AP, Affinity chromatography; MS, mass spectrometry; LC-MS/MS, liquid chromatography mass spectrometry; TIC, total ion current; m/z, mass/charge; CID, collision-induced dissociation; WB, Western blotting; IF immunofluorescence; Glucose regulated protein, GRP78 (accession # gi386758); heat shock 70kDa protein 8 isoform 1, HSP8, (accession # gi5729877); heat shock 70kDa protein 1, HSP1 (accession # gi4529893); Heat shock 70kDa protein 9, HSPA9 (accession # gi12653415).

FIGURE LEGENDS

Figure 1: LC-MS/MS analysis of a peptide mixture for identification of GRP78 protein as potential TDF-R. The TDF-P1 AP protein sample was separated on SDS-PAGE and the gel bands were excised and digested by trypsin. The resulting peptide mixture was separated on a C18 reverse phase column over an acetonitrile gradient. (A) TIC of the chromatogram. (B) MS survey mass spectrum, in which one double charged peak at m/z of 614.88 (expanded in the inbox) was fragmented by MS/MS and produced a MS/MS spectrum (C). The resulting peaks in the MS/MS spectrum correspond to a series of b and y ions that resulted from fragmentation of a peptide which was part of GRP78. Data analysis of these MSMS peaks led to identification of the sequence shown in (C). Data analysis of this MSMS spectrum, combined with data analysis of other MSMS spectra (data not shown) led to identification of GRP78 as potential TDF-R.

Figure 2: LC-MS/MS analysis of a peptide mixture for identification of HSP1 protein as potential TDF-R. The experimental procedure was performed as described in Figure 1. The TDF-P1 AP protein sample was separated on SDS-PAGE and the gel bands were excised and digested by trypsin. The resulting peptide mixture was separated on a C18 reverse phase column over an acetonitrile gradient. (A) TIC of the chromatogram. (B) MS survey mass spectrum, in which one double charged peak at m/z of 744.51 (expanded in the inbox) was fragmented by MS/MS and produced a MS/MS spectrum (C). The resulting peaks in the MS/MS spectrum correspond to a series of b and y ions that resulted from fragmentation of a peptide which was part of HSP1. Data analysis of these peaks led to identification of the sequence shown in (C). Data analysis of this MSMS spectrum, combined with data analysis of other MSMS spectra (data not shown) led to identification of HSP1 as potential TDF-R.

Figure 3: LC-MS/MS analysis of a peptide mixture for identification of HSP8 protein as potential TDF-R. The TDF-P1 AP protein sample was separated on SDS-PAGE and the gel bands were excised and digested by trypsin. The resulting peptide mixture was separated on a C18 reverse phase column over an acetonitrile gradient. (A) TIC of the chromatogram. (B) MS survey mass spectrum, in which one double charged peak at m/z of 472.83 (expanded in the inbox) was fragmented by MS/MS and produced a MS/MS spectrum (C). The resulting peaks in the MS/MS spectrum correspond to a series of b and y ions that resulted from fragmentation of a peptide which was part of HSP8. Data analysis of these peaks led to identification of the sequence shown in (C). Data analysis of this MSMS spectrum and other additional MSMS spectra (data not shown) led to identification of HSP8 as potential TDF-R.

Figure 4: LC-MS/MS analysis of a peptide mixture for identification of HSPA9 protein as potential TDF-R. The experimental procedure was performed as described in Figure 1. The TDF-P1 AP protein sample was separated on SDS-PAGE and the gel bands were excised and digested by trypsin. The resulting peptide mixture was separated on a C18 reverse phase column over an acetonitrile gradient. (A) TIC of the chromatogram. (B) MS survey mass spectrum, in which one double charged peak at m/z of 823.59 (expanded in the inbox) was fragmented by MS/MS and produced a MS/MS spectrum (C). The
resulting peaks in the MS/MS spectrum correspond to a series of b and y ions that resulted from fragmentation of a peptide which was part of HSPA9. Data analysis of these peaks led to identification of the sequence shown in (C). Data analysis of these peaks and other MSMS spectra (data not shown) led to identification of HSPA9 as potential TDF-R.

**Figure 5: WB of the affinity purified TDF-R candidates.** The TDF-R candidates were purified by AP using TDF-P1 peptide and the eluate investigated by WB using anti-GRP78 and anti-HSP70 antibodies. The cell lysate was prepared from MCF7 steroid-responsive cells (A), BT-549 steroid-resistant cells (B), HeLa cancer cells (C) and HDF-a cells (D). The molecular mass marker is shown (in kDa) on the left. Each WB contains input cell lysate (1), flow through (2) and eluate (3) of the AP experiments.

**Figure 6: IF detection of GRP78 protein.** Expression of GRP78 was investigated in steroid-responsive MCF7 and steroid-resistant BT-549 breast cancer cells, non-breast HeLa cancer cells and normal HDF-a. The cells were incubated with anti-GRP78 antibodies and then AlexaFluor 488 antibodies for detection of GRP78 protein (green). Plasma membrane was stained with CM-Dil (red) and nuclei were stained with DAPI (blue). The merged images are also shown. Enlarged images for each cell line are also shown.

**Figure 7: IF detection of HSP70 proteins.** Expression of HSP70 proteins was investigated in steroid-responsive MCF7 and steroid-resistant BT-549 breast cancer cells, non-breast HeLa cancer cells and normal HDF-a. The cells were incubated with anti-HSP70 antibodies and then AlexaFluor 488 antibodies for detection of HSP70 proteins (green). Plasma membrane was stained with CM-Dil (red) and nuclei were stained with DAPI (blue). The merged images are also shown.

**Figure 8: Immunoaffinity precipitation (IAP) of GRP78 and TDF-P1 mixture.** GRP78 protein was incubated with TDF-P1 for 2 hours and then the mixture was precipitated by IAP using anti-GRP78 antibodies (mouse monoclonal). The input, flow through, control and eluate were then investigated by WB using anti-GRP78 (rat monoclonal) and anti-TDF (rabbit polyclonal) antibodies. The molecular mass marker is shown (in kDa) on the left. Each WB contains input mixture of GRP78 protein and TDF-P1 (lane 1), flow through after IP (lane 2) negative control (lane 3) and eluate from IAP (lane 4).

**Table 1:** Summary of the proteins and peptides identified in our AP and MS experiments from MCF7 steroid-responsive breast cancer cells. In the control AP and MS experiments, where we used agarose beads without TDF-P1 peptide for AP, we identified the same Cytokeratins 8 and 18, known to be overexpressed in MCF7 cells.
Figure 1
Figure 2
Figure 3

A

B

C

[MH]^2+

TOF MS Survey ES+

TOF MSMS 472.83ES+

VCNPIITK

y2

b2

y6

y7

248.1765

628.4119

845.6020

260.1550

472.3179

686.5361

395.3097

473.3391

685.5212

473.3902

854.6020

627.8822

543.4014

544.4146

623.3621

578.3681

641e3

7.25e4

m/z

m/z

m/z

A

B

C

m/z
Figure 5

A

kDa 1 2 3
76

HSP70
GRP78
MCF7

B

kDa 1 2 3
76

HSP70
GRP78
BT-549

C

kDa 1 2 3
76

HSP70
GRP78
HeLa

D

kDa 1 2 3
76

HSP70
GRP78
HDF-a
Figure 6

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Figure 7

HSP70 | CM-Dil | DAPI | Merged
--- | --- | --- | ---
MCF7
BT-549
HeLa
HDF-a
Figure 8
Figure 9
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