Association of the DF3/MUC1 Breast Cancer Antigen with Grb2 and the Sos/Ras Exchange Protein

Pramod Pandey, Surender Kharbanda, and Donald Kufe

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Abstract

The human DF3/MUC1 gene encodes a high molecular weight mucin-like glycoprotein that is aberrantly overexpressed by breast cancer cells (1). Core proteins of the family of DF3-related antigens (also known as episialin, PEM) vary from Mr 160,000 to 230,000 (2). The demonstration that the DF3/MUC1 locus on chromosome 1q21-24 includes seven exons that span 4-7 kilobases (7-10). The NH2 terminal region of the protein core contains hydrophobic signal sequences that vary as a result of alternative splicing, whereas the COOH-terminal region includes a transmembrane domain and a cytoplasmic tail (9, 11-13). The 72-amino acid cytoplasmic domain also contains potential tyrosine phosphorylation sites that could bind to SH2 domains. The DF3/MUC1 antigen is detectable at elevated levels in the plasma of women with metastatic breast cancer and has been used to monitor their clinical course (14). Expression of this antigen has been shown to correlate with the degree of breast tumor differentiation and estrogen receptor status (15). The demonstration that the DF3/MUC1 antigen is also detectable in human milk (16) has supported the identification of a differentiation antigen that is aberrantly overexpressed by malignant mammary epithelium. Other studies have suggested that this family of glycoproteins regulates immune recognition and cellular adhesion (17, 18). The basis for these findings remains unclear and may be a function of the predicted rigid and extended structure of the glycoprotein.

The results of the present studies support involvement of the DF3/MUC1 protein in intracellular signaling. We demonstrate that tyrosine phosphorylation of the cytoplasmic tail is associated with binding of the SH2- and SH3-containing adaptor protein Grb2 (also known as ASH, Sem-5, and Drk, Refs. 19-22). The results also demonstrate that the DF3/Grb2 complex associates with the Ras activator protein Sos.

Materials and Methods

Cell Culture. Human MCF-7 breast carcinoma cells (Michigan Cancer Foundation, Detroit, MI) were grown as a monolayer in DMEM with 10% heat-inactivated fetal bovine serum, 100 units penicillin/ml, 100 μg streptomycin/ml, 2 mM l-glutamine, and 0.25 units insulin/ml.

Reagents. The mAb DF3 was prepared as described (1). Anti-Grb2 and anti-Sos antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-P-Tyr was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Immunoprecipitation and Immunoblotting. Lysates were prepared by suspending MCF-7 cells in lysis buffer [50 mM Tris (pH 7.6) 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 mM DTT, 10 mM sodium fluoride, 10 μg/ml of each leupeptin and aprotonin, and 1% Brij-96] for 30 min on ice. Lysates were cleared by centrifugation, and equal amounts of proteins (250-300 μg) were incubated with the indicated antibodies. Immune complexes were precipitated with protein A-Sepharose. The resulting precipitates were washed four times with lysis buffer and resolved by SDS-PAGE under reducing conditions. Proteins were then transferred to nitrocellulose, blocked by incubation in 5% nonfat dry milk in phosphate-buffered saline with 0.05% Tween-20 and probed with the indicated antibodies. The blots were then developed by ECL (Amersham, Arlington Heights, IL).

Fusion Protein-Binding Assays. The GST-Grb2 (full-length), GST-Grb2 SH2, GST-Grb2 N-SH3, and GST-Grb2 C-SH3 fusion proteins were purchased from Santa Cruz Biotechnology. Cell lysates were incubated with 20 μg immobilized GST or GST-Grb2 fusion proteins for 2 h at 4°C. The protein complexes were washed three times with lysis buffer and boiled for 5 min in SDS sample buffer. The complexes were then separated by 6% SDS-PAGE and subjected to silver-staining or immunoblot analysis with anti-DF3 or anti-Sos antibodies.

Peptide Synthesis and Competition Assays. Peptides were synthesized by using Fmoc-Tyr (P0,Me2)-OH for incorporation of phosphotyrosine and subsequently purified by ether precipitation and preparative reverse phase-HPLC. Amino acid analysis was used to confirm the sequence of the DF3-derived phosphopeptides: P1; SSLSpYTNPAV; and P2, TDRSpYEKVS. The GST-Grb2 SH2 protein was incubated in the presence or absence of 50 μM tyrosine-phosphorylated synthetic peptides at 4°C for 1 h. The fusion proteins were then incubated with cell lysates, and the adsorbates were analyzed by immunoblotting with anti-DF3.

Results and Discussion

The 72-amino acid cytoplasmic domain of the DF3 protein contains seven potential tyrosine phosphorylation sites that could interact with SH2 domains (Fig. 1A). SH2 domains bind to specific tyrosine-containing sequences in activated growth factor receptors and other phosphoproteins (23, 24). The specificity of these protein-protein interactions is dictated by the SH2 domain and the sequence surrounding

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The cytoplasmic tail of the DF3 protein contains a site (YTNP) that when phosphorylated on tyrosine could interact with the SH2 domain of the M, 26,000 Grb2 adaptor protein (Fig. 1A). Consequently, we asked whether DF3 associates with Grb2. Analysis of the anti-DF3 immunoprecipitates with anti-Grb2 demonstrated reactivity with a M, >200,000 protein (Fig. 1B). Moreover, immunoblotting of the mAb DF3 immunoprecipitate with anti-P-Tyr demonstrated that the DF3 antigen contains phosphorylated tyrosine residues (Fig. 1B).

The cytoplasmic domain of the DF3 protein contains seven potential tyrosine phosphorylation sites that could interact with SH2 domains. B and C. MCF-7 cell lysates were immunoprecipitated with either anti-DF3 antibody or preimmune rabbit serum (PIRS). The resulting immune complexes were analyzed by immunoblotting with either anti-DF3 or anti-P-Tyr antibodies. kD, molecular weight in thousands.

To confirm an association between Grb2 and DF3, we boiled anti-Grb2 immunoprecipitates in 0.5% SDS buffer to disrupt protein-protein interactions and then subjected the proteins to secondary immunoprecipitations using anti-DF3 or preimmune rabbit serum. Analysis of the secondary immunoprecipitates by immunoblotting with anti-DF3 confirmed binding of Grb2 and DF3 (Fig. 2C). The association between DF3 and Grb2 was further examined by using GST-Grb2 fusion proteins. Lysates from MCF-7 cells were incubated with GST, GST-Grb2 (full-length), GST-Grb2 SH2, and GST-Grb2 N-SH3. Analysis of the adsorbates by immunoblotting with anti-DF3 revealed binding of DF3 to GST-Grb2 (full-length) and GST-Grb2 SH2. In contrast, there was no detectable binding to GST, GST-Grb2 N-SH3, and GST-Grb2 C-SH3 (Fig. 3A; data not shown). These findings indicated that DF3 selectively interacts with the SH2 domain of Grb2. To define the site in DF3 responsible for the association with Grb2, we identified two potential candidate sequences, YTNP and YEKV (Fig. 1A). The SH2 domain of Grb2 binds to the consensus sequence pYXNP/V. Chemically phosphorylated synthetic peptides corresponding to pYTNP and pYEKV were used in competition assays. Preincubation of GST-Grb2 SH2 with pYTNP inhibited binding of DF3 from lysates of MCF-7 cells, although there was no detectable inhibition of DF3 binding when using pYEKV as a control.
**INTERACTION OF DF3 WITH Grb2/Sos**

**A.**

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**B.**

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**C.**

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Fig. 4. MCF-7 cell lysates were subjected to affinity chromatography with GST, GST-Grb2, GST-Grb2 SH2, and GST-Grb2 N-SH3 fusion proteins. The resulting protein complexes were analyzed by immunoblotting with anti-Sos. C. MCF-7 cell lysates were immunoprecipitated with anti-DF3 or anti-Sos antibodies. The resulting immune complexes were analyzed by immunoblotting with anti-Sos or anti-DF3. kD, molecular weight in thousands.

**References**


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