Nerve Growth Factor Receptor TrkA Signaling in Breast Cancer Cells Involves Ku70 to Prevent Apoptosis*

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The nerve growth factor (NGF)-tyrosine kinase receptor TrkA plays a critical role in various neuronal and non-neuronal cell types by regulating cell survival, differentiation, and proliferation. In breast cancer cells, TrkA stimulation results in the activation of cellular growth, but downstream signaling largely remains to be described. Here we used a proteomics-based approach to identify partners involved in TrkA signaling in breast cancer cells. Wild type and modified TrkA chimeric constructs with green fluorescent protein were transfected in MCF-7 cells, and co-immunoprecipitated proteins were separated by SDS-PAGE before nano-LC-MS/MS analysis. Several TrkA putative signaling partners were identified among which was the DNA repair protein Ku70, which is increasingly reported for its role in cell survival and carcinogenesis. Physiological interaction of Ku70 with endogenous TrkA was induced upon NGF stimulation in non-transfected cells, and co-localization was observed with confocal microscopy. Mass spectrometry analysis and Western blotting of phosphotyrosine immunoprecipitates demonstrated the induction of Ku70 tyrosine phosphorylation upon NGF stimulation. Interestingly no interaction between TrkA and Ku70 was detected in PC12 cells in the absence or presence of NGF, suggesting that it is not involved in the initiation of neuronal differentiation. In breast cancer cells, RNA interference indicated that whereas Ku70 depletion had no direct effect on cell survival, it induced a strong potentiation of apoptosis in TrkA-overexpressing cells. In conclusion, TrkA signaling appears to be proapoptotic in the absence of Ku70, and this protein might therefore play a role in the long time reported ambivalence of tyrosine kinase receptors that can exhibit both anti- and eventually proapoptotic activities.

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Nerve growth factor (NGF) is the prototypic member of the neurotrophin family of proteins that is mainly known for promoting survival and differentiation of neuronal cells during nervous system development (1). NGF neurotrophic activities are mediated through two membrane receptors, the tyrosine kinase receptor TrkA and the receptor p75NTR, a member of the tumor necrosis factor (TNF) receptor superfamily. NGF binding to its receptors leads to a variety of intracellular signaling events in neuronal cells (2), starting with dimerization and activation of the tyrosine kinase activity of TrkA and its subsequent transphosphorylation, which creates docking sites for adaptor proteins leading to the activation of intracellular signaling cascades. Among described signaling cascades are the Ras/ERK/mitogen-activated protein (MAP) kinase, the phosphatidylinositol 3-kinase (PI3K)/Akt kinase, and the phospholipase C-γ1 pathways.

Aside from neurotrophism, other NGF activities have been described in non-neuronal cell types, and the involvement of this growth factor in oncogenesis of neuronal and non-neuronal origin has been documented. This is particularly the case in brain, ovarian, prostatic, pancreatic, lung, and breast cancers in which NGF production has been reported and eventually impacts tumor cell growth (2–7). In breast cancer, NGF is overexpressed and acts as a paracrine/autocrine factor to enhance tumor cell growth and survival (8–10). TrkA and p75NTR are expressed in the majority of breast tumors and are related to prognosis of breast tumors (11–13). Interestingly TrkA cooperates with the membrane tyrosine kinase p185 (HER2) to activate the proliferation of breast cancer cells (14), and the suppression of TrkA activity by endocannabinoids results in the inhibition of breast cancer cell growth (15). Importantly the reference drug used in hormone therapy of breast cancer, tamoxifen, inhibits the proliferative effect of NGF in breast cancer (16), indicating that targeting NGF sig-

1 The abbreviations used are: NGF, nerve growth factor; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; sRNA, small interfering RNA; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; VCP, valosin-containing protein; MAP, mitogen-activated protein; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase.

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naling in breast cancer is of potential interest for the development of future therapeutic approaches.

To date, TrkA signaling has not been extensively described in breast cancer cells. It is known that TrkA activation leads to a stimulation of cell proliferation that is mediated by the MAP kinase pathway (9, 16), and a better knowledge of the signaling molecules involved would be important for a better understanding of mammary oncogenesis and the identification of new molecular targets. In the present work, we used a proteomics strategy to decipher the TrkA signaling pathway in MCF-7 breast cancer cells. TrkA-green fluorescent protein (GFP) constructs were used to co-immunoprecipitate proteins that interact with TrkA, and these proteins were separated by SDS-PAGE before analysis with nano-LC-MS/MS. 13 TrkA putative interacting partners were identified among which was the Ku70 protein initially described as a regulatory subunit of the DNA-dependent protein kinase that is crucial to DNA repair. Ku70 is important for the control of cell survival, particularly in proliferating cells in which DNA synthesis must be accompanied by efficient DNA repair, and the implication of Ku70 in oncogenesis is increasingly reported (17). In the present study, the involvement of Ku70 in TrkA signaling pathway was revealed, and our results suggest its implication in the control of TrkA activity and survival of breast cancer cells.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Bio-Whittaker. Rat tail type I collagen was obtained from Upstate. Recombinant NGF was from R&D Systems. Nucleobond plasmid DNA purification kit was purchased from Machery-Nagel. Nucleofection reagents were from Amaxa Biosystems. Dynabeads protein A and protein G were obtained from Dynal Biotech. Electrophoresis reagents, bicinechonic acid reagents, protease inhibitor mixture, and rabbit polyclonal anti-actin antibody were from Sigma. Sequencing grade modified trypsin was provided by Promega. ZipTipC18 pipette tips were obtained from Millipore. The rabbit polyclonal anti-GFP antibody (BD Living Colors™ Full-Length) and the mouse monoclonal anti-Ku70 IgG1 (clone 15) were purchased from BD Biosciences. The mouse monoclonal anti-GFP antibody (BD-2) and anti-phosphotyrosine residue (pY99) antibodies were from Santa Cruz Biotechnology, Inc. The rabbit polyclonal anti-TrkA IgG was from Upstate, and the rabbit polyclonal anti-Ku70 antiserum (AHP316) was from Serotec. Peroxdase-conjugated donkey anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories. SuperSignal West Pico Chemiluminescent Substrate was from Pierce. Lab-Tek chamber slides were obtained from Nalge Nunc International. Alexa Fluor dye-conjugated secondary antibodies were from Invitrogen. siRNAs were purchased from Eurogentec.

Cell Culture and NGF Stimulation—The MCF-7 human breast cancer cell line and the PC12 rat adrenal pheochromocytoma cell line were purchased from the American Type Culture Collection. MCF-7 cells were routinely maintained in minimum essential medium Eagle’s balanced salt solution supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% fetal calf serum, 40 units/ml penicillin-streptomycin, 40 μg/ml gentamycin, and 10 μg/ml insulin. PC12 cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 2 mM L-glutamine, 10% fetal calf serum, 5% horse serum, and 20 units/ml penicillin-streptomycin. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. NGF stimulation of MCF-7 and PC12 cells was performed after 16 h of serum starvation by addition of 200 ng/ml NGF for 10 min.

Plasmid and siRNA Transfection—TrkA-GFP chimeras were constructed as described previously (18). Plasmid DNA purification was carried out using the Nucleobond kit according to the manufacturer’s instructions. MCF-7 cells were transiently transfected using the nucleofection technology according to the Amaxa Biosystems protocol. Briefly 2 × 10⁶ cells were resuspended in 100 μl of Cell Line Nucleofector™ Solution V, and the cell suspension was mixed with 2 μg of TrkA-GFP vector. The sample was transferred into an electroporation cuvette, and transfection was performed using the program E-14 according to the manufacturer’s instructions. Immediately after nucleofection, cells were transferred into prewarmed complete maintenance medium and were cultured as described before.

siRNAs against Ku70 (sense, 5'-GAUGCCUUACUGAAAAA-3'; antisense, 5'-UUUUCGAUGAAAGGCAU-3') were those defined and tested previously by Ayene et al. (19). MCF-7 cells were transiently nucleofected with 3 μg of siRNA directed against Ku70 as described above.

Determination of Apoptotic Cells—24 h after transfection, cells were deprived in a serum-free medium for 12 h, stimulated with 200 ng/ml NGF for 1 h, and treated with 200 ng/ml NGF and 5 ng/ml TNF-related apoptosis-inducing ligand (TRAIL) for 6 h. Apoptosis was determined by morphological analysis after fixation with methanol (10 min at −20 °C) and staining with 1 μg/ml Hoechst 33258 (10 min at room temperature in the dark). A minimum of 500–1000 cells was examined for each case under a fluorescence microscope, and the results represented the number of apoptotic cells over the total number of counted cells.

Protein Extraction, Immunoprecipitation, and SDS-PAGE—Cells were harvested by scraping in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, and protease inhibitor mixture). After centrifugation (10,000 × g for 3 min at 4 °C), the supernatant proteins were quantified using the bicinchoninic acid method. TrkA-GFP and interacting proteins were co-immunoprecipitated from 5 mg of proteins using 25 μg of anti-GFP antibody (BD Living Colors Full-Length) and Dynabeads protein A according to the manufacturers’ instructions. For the immunoprecipitation of phosphorylated proteins, 5 mg (for MS identification) or 1 mg (for immunoblot detection) of proteins was used with 25 or 5 μg, respectively, of anti-phosphotyrosine antibody (pY99) and Dynabeads protein G. The endogenous TrkA immunoprecipitation was performed from 1 mg of proteins using 5 μg of anti-TrkA and Dynabeads protein A. Immunoprecipitated proteins were eluted in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue) and boiled for 5 min before analysis on 10% polyacrylamide gels. For colloidal Coomassie staining (20), SDS-polyacrylamide gels were fixed overnight in solution containing 50% ethanol and 1.4% orthophosphoric acid. After three washes for 30 min in MilliQ water, gels were incubated in impregnation solution (1.3 mM ammonium sulfate, 34% methanol, and 1.4% orthophosphoric acid) for 1 h and placed in staining solution (1.3 mM ammonium sulfate, 34% methanol, 1.4% orthophosphoric acid, and 0.07% Coomassie Brilliant Blue G250) for 24 h. Finally gels were destained with several washes of MilliQ water until the background was clear.

In-gel Trypsin Digestion—Coomassie Blue-stained protein bands were excised from the SDS-PAGE gel and processed for trypsin digestion as described previously (21) with minor modifications. In brief, gel bands were destained twice for 10 min in a solution containing 100 mM NH₄HCO₃ and 50% acetonitrile, dehydrated in acetonitrile, and dried in a vacuum centrifuge. Gel pieces were then rehydrated at 4 °C for 45 min in a digestion buffer (50 mM NH₄HCO₃ and 12.5 ng/μl trypsin). The supernatant was replaced by 40 μl of 50
mM NH₄HCO₃, and the samples were incubated overnight at 37 °C. The tryptic peptides were recovered by 10-min incubations, once in 25 mM NH₄HCO₃ and 50% acetonitrile and twice in 25 mM NH₄HCO₃, 50% acetonitrile, and 5% formic acid. All supernatants were pooled and dried in a vacuum centrifuge.

**Protein Identification by Mass Spectrometry**—For MALDI-TOF analyses, tryptic digests were resuspended in 1% formic acid and were desalted and concentrated using ZipTip-C₁₈ pipette tips according to the manufacturer’s instructions. Peptides were spotted on the MALDI target with 10 mg/ml dihydroxybenzoic acid in 50% acetonitrile and 5% formic acid. All supernatants were pooled for MALDI-TOF mass spectra were acquired using a Voyager DE STR instrument (Applied Biosystems) in reflector positive ion mode. For mass fingerprint analysis, each raw spectrum was opened in Voyager Data Explorer software (version 4.6) and treated using advanced base-line correction and noise removal 2 functions. It was calibrated with two peptides resulting from trypsin autolysis (m/z 842.5100 and 2211.1046). The filter peak list for monoisotopic masses only was enabled, the peak detection threshold was manually adjusted over the background, and then the peak list was copied to the Mascot public interface (version 2.1) and searched against the Swiss-Prot database 51.3 (250,293 sequences; 91,444,238 residues) using the following parameters: trypsin as enzyme, two possible missed cleavages, oxidized methionine as variable modification, MH⁺ monoisotopic masses, and peptide tolerance of 70 ppm. Results were scored using the probability-based Mowse score (The protein score is \( p \) where \( p \) is the probability that the observed match is a random event. Protein scores greater than 66 are significant \( p < 0.05 \).) Nano-LC-nano-ESI-MS/MS analyses were performed on an ion trap mass spectrometer (LCQ Deca XP+, Thermo Electron Corp.) equipped with a nanoelectrospray ion source coupled with a nano high pressure liquid chromatography system (LC Packings Dionex). Tryptic digests were resuspended in 4 μl of 0.1% HCOOH, and 1.4 μl were injected into the mass spectrometer using the Famos autosampler (LC Packings Dionex). The sample was first desalted and then concentrated on a reverse phase precolumn of 5 mm × 0.3-mm inner diameter (Dionex) by solvent A (95% H₂O, 5% acetonitrile, 0.1% HCOOH) delivered by the Switchos pumping device (LC Packings Dionex) at a flow rate of 10 μl/min for 3 min. Peptides were separated on a 15-cm × 75-μm-inner diameter, 3-μm, C₁₈ PepMap column from Dionex. The flow rate was set at 200 nl/min. Peptides were eluted using a 5–70% linear gradient of solvent B in 45 min (25% H₂O, 80% acetonitrile, 0.08% HCOOH). Coated nanoelectrospray needles (560-μm outer diameter, 20-μm inner diameter, 10-μm tip inner diameter, standard coating) were obtained from New Objective (Woburn, MA). Spray voltage was set at 1.5 kV, and capillary temperature was set at 170 °C. The mass spectrometer was operated in positive ionization mode. Data acquisition was performed in a data-dependent mode consisting of alternately in a single run a full scan MS over the range m/z 2000–500 and a full scan MS/MS of the ion selected in an exclusion dynamic mode (the most intense ion was selected and excluded for further selection for a duration of 3 min). MS/MS data were acquired using a 2 m/z unit ion isolation window and a 35% relative collision energy. MS/MS .raw data files were transformed in .dta files with Bioworks 3.1 software (Thermo Electron Corp.). The .dta files generated were next merged with merge.bat software to be downloaded in Mascot software (version 2.1) for database searches in Swiss-Prot 51.3 (250,293 sequences; 91,444,238 residues). Search parameters were the following: *Homo sapiens* for taxonomy, one allowed missed cleavage, methionine oxidation as variable modification, 2 Da for peptide tolerance, and 0.8 Da for MS/MS tolerance. Results were scored using the probability-based Mowse score (The protein score is \( -10 \log(p) \) where \( p \) is the probability that the observed match is a random event. Protein scores greater than 66 are significant \( p < 0.05 \).) To ascertain unambiguous identification, searches were performed in parallel with Phenyx software using the same parameters. It should be noted that a protein can appear in databases under different names and accession numbers. In addition, because a shared sequence may represent a problem, a minimum of two peptides with an individual score of at least 40 were considered for identification, and all sequences obtained by MS/MS analysis were checked using the Basic Local Alignment Search Tool (BLAST) public interface (version 2.2.13) to exclude that sequence sharing with other proteins could interfere with the reliability of the identification.

**Western Blot**—After separation on SDS-PAGE, co-immunoprecipitated proteins were electrotransferred onto nitrocellulose membrane using a semidry transfer system (Trans-Blot SD cell, Bio-Rad). Efficiency of transfer and relative equal loading was checked with Ponceau S staining. Nonspecific protein binding sites were saturated for 1 h at room temperature in TBS, 0.1% Tween 20 reagent (TBST) containing either 5% nonfat dry milk for Ku70 immunodetection, 3% nonfat dry milk for TrkA immunodetection, or 5% BSA for GFP and actin immunodetection. Membranes were then incubated overnight at 4 °C with 1:5000 anti-Ku70 (AHP316), 1:1000 anti-Ku70 (clone 15), 1:500 anti-TrkA, 1:1000 anti-GFP (B-2), or 1:5000 anti-actin. After washes in TBST, peroxidase-conjugated anti-rabbit or anti-mouse IgG diluted in saturated solution was added for 1 h at room temperature, and the membranes were washed several times in TBST before detection of peroxidase activity using a chemiluminescence system.

**Immunocytochemistry and Confocal Microscopy**—MCF-7 cells were seeded on a Lab-Tek chamber slide system precoated with 1% type I collagen. Cells were washed in PBS, pH 7.5; fixed in 4% paraformaldeyde for 20 min; and permeabilized in PBS, pH 7.5, containing 0.05% saponin and 50 mM ammonium chloride. Nonspecific protein binding sites were then blocked in PBS, pH 7.5, containing 0.05% saponin and 2% BSA, and cells were incubated in blocking solution containing 10 μg/ml rabbit anti-TrkA and 10 μg/ml mouse anti-Ku70 (clone 15) antibodies overnight at 4 °C. After washes in PBS, pH 7.5, 10 μg/ml Alexa Fluor 546-conjugated goat anti-rabbit IgG and 10 μg/ml Alexa Fluor 488-conjugated donkey anti-mouse IgG were added for 1 h at 37 °C. Cells were then washed in PBS, pH 7.5, and mounted in Mowiol®. Scanning fluorescence images were acquired using a Zeiss Axioshot microscope.

**RESULTS**

**Identification of TrkA Co-immunoprecipitated Proteins**—Human MCF-7 cells were transiently transfected with TrkA-GFP construct ∆D, corresponding to the full-length TrkA, or the ∆(8 construct lacking functional intracellular domain of TrkA (Fig. 1A). The full-length TrkA-GFP chimera was used in the first step of our experiments to increase the amount of TrkA and to have a bait to co-immunoprecipitate TrkA and its signaling protein complex. TrkA-GFP constructs were well characterized, and the full-length chimera has the same biological properties as the endogenous TrkA, such as the tyrosine phosphorylation after NGF stimulation, receptor internalization and trafficking, the activation of MAP kinase, and the differentiation of PC12 cells (18). Moreover we used a TrkA-GFP construct with a truncated intracellular domain of the receptor lacking the tyrosine kinase domain and tyrosine-based signaling motifs Tyr⁹⁹⁹, Tyr⁶⁷⁹/⁶⁸³, and Tyr⁷⁹⁴ so that it is unable to bind downstream signaling partners and to transduce NGF signaling (18). After NGF stimulation, cell lysates were subjected to GFP immunoprecipitation, and elu-
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MALDI-TOF and MS/MS allowed the identification of the two proteins derived from the TrkA-GFP constructs (Table I). For Δ0 (TrkA-GFP full length), both peptide mass fingerprint and sequence information on the 1403.70-Da peptide were displayed in Fig. 2A. The analysis of the MALDI-TOF spectrum showed 21 of 24 experimental tryptic peptides were matched for the identification by peptide mass fingerprint of TrkA-GFP, leading to 14% sequence coverage with an average error mass of 51 ppm (Table I) and a score of 116. MS/MS sequence information on two peptides for TrkA (1403.7 and 1517.77 Da) and four peptides for GFP (1346.65, 1476.76, 1502.65, and 1541.78 Da) strengthened this identification.

For the identification of TrkA-GFP co-immunoprecipitated proteins, the nano-LC-nano-ESI ion trap analyses of SDS-PAGE bands led to the identification of 13 putative signaling partners from 53 peptide sequences. A large number of peptides were not assigned to a protein based on our stringent criteria for identification; proteins with low probabilistic value were omitted. Thus, the proteins that we report here were unambiguously identified (Table II). The confidence of the identification was expressed by the Mascot score and the expected value of each peptide (p < 0.01). The list of co-immunoprecipitated proteins includes GTPase activator (Ras GTPase-activating-like protein IQGAP1), heterogeneous nuclear ribonucleoprotein U, valosin-containing protein (ATPase), the elongation factor 2 (EF-2), the 78-kDa glucose-related protein (GRP78), the heat shock cognate 71-kDa protein, the DNA repair protein Ku70, the ATP-dependent RNA helicase DDX5, actin, interleukin enhancer-binding factor-2, nucleophosmin, proliferating cell nuclear antigen (PCNA), and fibrillarin. The known molecular function of each protein, according to the Human Protein Reference Database (www.hprd.org), is indicated (Table II). Among the identified proteins, the DNA repair protein family Ku70 was intriguing. Ku70 is increasingly appearing as being involved in carcinogenesis, and it has not been reported to interact with TrkA or participate in its downstream signaling. DNA repair is required during cell proliferation, otherwise leading to cell death. Because NGF stimulates both proliferation and survival of breast cancer cells, the identification of Ku70 as a TrkA co-immunoprecipitated protein (i.e. putative signaling partner) was of particular interest, and further experiments, described below, were designed to explore the involvement of Ku70 in TrkA signaling and cellular responses.

Ku70 Interaction with TrkA Is Induced by NGF Stimulation—To validate the interaction between endogenous TrkA and Ku70 in physiological conditions, we first investigated the possible cellular co-localization of these two proteins in non-transfected breast cancer cells (Fig. 3A). In confocal microscopy, TrkA (red fluorescence) was mainly located in the cytoplasm and cytoplasmic membrane (arrow in row TrkA) of...
MCF-7 cells but also observed slightly in the nucleus (arrowhead in row TrkA). This localization corresponded to what has previously been reported in other cell types (18), and in addition, nuclear translocation of tyrosine kinase receptors is increasingly described (22). Concerning Ku70, green fluorescence was observed in the nucleus (arrowhead in row Ku70) with a weaker localization in the cytoplasm (arrow in row Ku70). The overall co-localization of TrkA and Ku70 in both the cytoplasm and the nucleus of MCF-7 cells was reflected by the yellow-orange merge fluorescence (arrowhead and arrow in row Merge). In conclusion, the cellular localization of TrkA and Ku70 that we observed in confocal microscopy is compatible with an interaction of these two proteins. The interaction of TrkA and Ku70 was further studied after immunoprecipitation from nontransfected MCF-7 cells followed by a Western blot immunodetection of Ku70 (Fig. 3B). The results validated Ku70 co-immunoprecipitation with TrkA and indicated that this was observed only upon cell stimulation with NGF, whereas it was not observed in control unstimulated cells. Therefore, the interaction between TrkA and Ku70 in breast cancer cells is induced by NGF stimulation. Interestingly no interaction between TrkA and Ku70 was detected in the neuronal-like PC12 cells indicating a differential regulation between neuronal and non-neuronal cell types and a non-involvement of Ku70 in TrkA signaling leading to cell differentiation.

Ku70 Is Tyrosine Phosphorylated upon NGF Stimulation—Tyrosine phosphorylation is the prime post-translational modification initiated by TrkA signaling. The immunoprecipitation of proteins phosphorylated on tyrosine in response to NGF stimulation in breast cancer cells was performed using phosphotyrosine-specific antibody (pY). A TrkA immunoblot following the pY immunoprecipitation showed the activation of

| MH Da | Sequence |
|-------|----------|
| 845.46 | 845.48 |
| 882.46 | 882.41 |
| 898.40 | 898.40 |
| 1093.52 | 1093.52 |
| 1118.51 | 1118.57 |
| 1195.77 | 1195.52 |
| 1291.69 | 1291.64 |
| 1404.76 | 1404.70 |
| 1518.87 | 1518.77 |
| 1818.09 | 1817.96 |
| 1833.97 | 1833.92 |
| 2031.10 | 2031.04 |
| 821.36 | 821.39 |
| 1050.56 | 1050.51 |
| 1266.60 | 1266.57 |
| 1282.57 | 1282.57 |
| 1347.62 | 1347.65 |
| 1477.80 | 1477.76 |
| 1503.70 | 1503.65 |
| 1973.88 | 1973.90 |
| 1989.87 | 1989.89 |
| 845.48 | 845.48 |
| 1404.76 | 1404.7 |
| 1518.84 | 1518.77 |
| 2031.13 | 2031.04 |
| 2474.44 | 2474.2 |
| 821.42 | 821.39 |
| 1050.52 | 1050.51 |
| 1266.63 | 1266.57 |
| 1282.5 | 1282.57 |
| 1347.6 | 1347.65 |
| 1477.81 | 1477.76 |
| 1503.55 | 1503.65 |
| 1542.68 | 1542.78 |
| 1973.79 | 1973.9 |
| 1989.88 | 1989.89 |

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TrkA after NGF stimulation (Fig. 4A). After separation by SDS-PAGE, pY-immunoprecipitated proteins were visualized. A 72-kDa band, corresponding to the molecular mass of Ku70, was specifically detected in NGF-stimulated MCF-7 cells (Fig. 4A, upper panel) and was subjected to peptide mass fingerprint identification. The MALDI-TOF analysis (Fig. 4B) of the tryptic digestion peptides indicated that 19 of 40 peptides matched the theoretical tryptic peptides of the Ku70 protein with a high accuracy (32 ppm), covering 39% of its sequence (Fig. 4C) with a score of 148. In addition, the immunoprecipitation of phosphotyrosine proteins in MCF-7 cells with or without NGF treatment followed by Ku70 immunodetection by Western blot revealed a band corresponding to phosphorylated Ku70 only in NGF-stimulated MCF-7 cells (Fig. 4D). Interestingly, no phosphorylation of Ku70 was induced in PC12 cells upon NGF stimulation. These results indicated that NGF stimulation of breast cancer cells resulted in the induction of Ku70 tyrosine phosphorylation.

Ku70 Is Involved in TrkA Signaling and Survival of Breast Cancer Cells—It has been reported that Ku70 can prevent cell death (23), and we have shown previously that NGF stimulates the survival and proliferation of breast cancer cells (9). Because an interaction between TrkA and Ku70 was identified, we explored the possibility of a regulation of Ku70 antiapoptotic activity by TrkA. In our experiments, we induced breast cancer cell death using the proapoptotic agent TRAIL. The data presented in Fig. 5A show that whereas RNA interference against Ku70 had no proapoptotic effect by itself it induced a strong potentiation of TRAIL-induced apoptosis in breast cancer cells overexpressing TrkA. Condensation and fragmentation of the nucleus, characteristics of apoptosis, can be observed in Fig. 5B, particularly in the situation of Ku70 depletion by specific siRNA. Because there is no potentiation of TRAIL-induced apoptosis when TrkA is not overexpressed, we hereby propose Ku70 as a new player involved in TrkA-mediated survival...
TABLE II
Identification of TrkA co-immunoprecipitated proteins

GFP co-immunoprecipitated proteins were separated by SDS-PAGE (Fig. 1B), and bands differentially found between Δ0 (TrkA-GFP full length) and Δ8 (TrkA-GFP deleted from TrkA intracellular domain) were cut out of the gel and digested with trypsin before nano-LC-nano-ESI-MS/MS analysis. Band number, protein name, Swiss-Prot accession number, peptide sequences, the Mascot score, and the percentage of coverage are reported for each protein. The observed precursor m/z, the theoretical precursor neutral mass, the delta mass in Da, and the expected value (p < 0.01) delivered by the Mascot search program are indicated for each peptide. The molecular function of each protein, according to the Human Protein Reference Database (www.hprd.org), is indicated. Mox indicates a methionine oxidation. Representative MS/MS spectra of each identified proteins are shown in the supplemental data.

| Band number | Protein name | Swiss-Prot accession no. | Peptide sequences | Observed precursor m/z | Theoretical precursor neutral mass | Delta mass | Expected value | Mascot score | Percentage of coverage | Molecular function |
|-------------|--------------|--------------------------|------------------|------------------------|-----------------------------------|-----------|---------------|--------------|------------------------|-------------------|
| 1           | Ras GTPase-activating-like protein IQGAP1 | P46940 | TLQALQIPAALK | 577.36 | 1152.69 | 0.01 | 5.9e-05 | 213 | 2 | GTPase activator |
|             |              |                          | ATFYGEQVDYYK    | 742.33 | 1482.67 | -0.02 | 1.8e-05 |               |                       |
|             |              |                          | SNQQLENDNLMDIK | 896.00 | 1789.85 | 0.14 | 2.8e-10 |               |                       |
| 2           | Heterogenous nuclear ribonucleoprotein U | Q00839 | FIEAAR | 819.35 | 1559.67 | -0.12 | 3.0e-01 | 283 | 9 | Ribonucleoprotein |
|             |              |                          | NGDGLGVAFK      | 525.50 | 1053.97 | 1.45 | 2.5e-02 |               |                       |
|             |              |                          | GYFEYIENK      | 646.59 | 1290.58 | 0.59 | 7.6e-03 |               |                       |
|             |              |                          | YNILGNTIMDK Mox | 700.04 | 1397.69 | 0.39 | 5.7e-04 |               |                       |
|             |              |                          | NFILDQTNVAAQR  | 824.60 | 1647.85 | 0.34 | 1.9e-04 |               |                       |
|             |              |                          | GYFEYIENKYSR   | 849.65 | 1695.87 | 0.52 | 2.5e-03 |               |                       |
|             |              |                          | SSSGPTSLFAVTVAPPGAR | 858.01 | 1713.90 | 0.09 | 1.0e00 |               |                       |
| 3           | Valosin-containing protein | P55072 | EMVELPLR Mox | 501.83 | 1001.67 | 0.12 | 5.0e00 | 176 | 6 | ATPase activity |
|             |              |                          | LEILQHTK       | 547.91 | 1093.83 | 0.16 | 8.7e-04 |               |                       |
|             |              |                          | GILYGGPGTGK    | 586.74 | 1171.48 | -0.20 | 3.2e-03 |               |                       |
|             |              |                          | GDIFLVRGGMR Mox | 619.83 | 1235.59 | 1.99 | 3.7e01 |               |                       |
|             |              |                          | LDQLIYIPLPDEK  | 773.96 | 1547.88 | 0.87 | 6.3e-03 |               |                       |
|             |              |                          | NFILDQTNVAAQR  | 824.60 | 1647.85 | 0.34 | 1.9e-04 |               |                       |
| 4           | 78-kDa glucose-regulated protein | P11021 | DAGTIALNVMR Mox | 617.64 | 1232.62 | 0.65 | 5.6e-03 | 523 | 19 | Chaperone |
|             |              |                          | NELESYAASLK   | 658.97 | 1315.63 | 0.29 | 1.8e00 |               |                       |
|             |              |                          | TWNDPSVQDIK    | 716.02 | 1429.68 | 0.35 | 2.8e-03 |               |                       |
|             |              |                          | ITPSYVAFTPTEGER | 783.98 | 1565.77 | 0.18 | 2.6e-03 |               |                       |
|             |              |                          | KSDIDEIVLVGGSTR | 795.19 | 1587.89 | 0.52 | 8.1e-09 |               |                       |
|             |              |                          | TKPYQDVGQGGQT | 803.11 | 1603.86 | 0.35 | 1.3e-04 |               |                       |
|             |              |                          | NQLTSPENTVFDAK | 839.55 | 1676.80 | 0.28 | 5.4e-06 |               |                       |
|             |              |                          | INEPTAAIAAYGLDK | 908.65 | 1814.99 | 0.30 | 3.1e-05 |               |                       |
|             |              |                          | SQIFSTASNDNQPTVTIK | 919.34 | 1835.93 | 0.73 | 7.3e-06 |               |                       |
| 5           | Heat shock cognate 71-kDa protein | P11142 | DAGTIALNLVR | 600.60 | 1197.67 | 0.32 | 2.8e-02 | 554 | 18 | Heat shock protein |
|             |              |                          | MKNHFAEFK Mox | 626.55 | 1250.61 | 0.47 | 9.3e-03 |               |                       |
|             |              |                          | MKIEAEAYLGGK Mox | 635.10 | 1267.65 | 0.54 | 6.6e-03 |               |                       |
|             |              |                          | NSLESYAFNMMK Mox | 660.49 | 1318.59 | 0.38 | 9.1e-05 |               |                       |
|             |              |                          | SQHDDILVGGSTR  | 741.88 | 1480.80 | 0.94 | 1.5e-04 |               |                       |
|             |              |                          | TTPSYAFTDTER  | 744.35 | 1486.69 | 0.03 | 4.6e-03 |               |                       |
|             |              |                          | SFYPEEVSVMVTIK Mox | 816.91 | 1631.77 | 0.02 | 1.5e-04 |               |                       |
|             |              |                          | INEPTAAIAAYGLDK | 830.67 | 1658.89 | 0.43 | 4.2e-05 |               |                       |
|             |              |                          | NOQVAMNPTNTVFDAK Mox | 833.48 | 1664.78 | 0.15 | 1.4e-05 |               |                       |
|             |              |                          | INEPTAAIAAYGLDKK | 894.69 | 1786.98 | 0.38 | 3.7e-07 |               |                       |
| Band number | Protein name          | Swiss-Prot accession no. | Peptide sequences                  | Observed precursor m/z | Theoretical precursor neutral mass | Delta mass Da | Expected value | Mascot score | Percentage of coverage % | Molecular function                        |
|-------------|-----------------------|--------------------------|------------------------------------|------------------------|-----------------------------------|---------------|---------------|--------------|--------------------------|-------------------------------------------|
| 6           | Ku70 (ATP-dependent DNA helicase) | P12956                   | SDSFENPVLOQQHFR                    | 568.86                 | 1702.81                           | 0.76          | 5.9e-03       | 133          | 8                        | DNA repair                                |
|             |                       |                          | IMLFTNEDNPHGNDSAK Mox              | 640.78                 | 1917.85                           | 1.47          | 1.5e-05       |             |                          |                                           |
|             |                       |                          | TEGDEEAEEEQEENLEASGDYK             | 1251.20                | 2499.99                           | 0.39          | 2.4e-01       |             |                          |                                           |
| 6           | Probable ATP-dependent RNA helicase DDX5 | P17844                   | KWNDELPK                            | 572.52                 | 1141.61                           | 1.41          | 2.9e00        | 127          | 7                        | RNA binding                               |
|             |                       |                          | TTYLVLDEADRR                        | 648.66                 | 1294.64                           | 0.66          | 1.0e00        |             |                          |                                           |
|             |                       |                          | NFYOEHPSLAR                         | 695.60                 | 1388.65                           | 0.54          | 3.6e-02       |             |                          |                                           |
|             |                       |                          | TGATAYFFTPNNIK                      | 787.94                 | 1573.78                           | 0.09          | 7.0e-04       |             |                          |                                           |
| 7           | Actin β/γ              | P60709                   | GILTLK                              | 644.41                 | 643.43                            | -0.02         | 3.3e00        | 294          | 21                       | Structural constituent of cytoskeleton    |
|             |                       |                          | GYSFTTTAER                          | 567.20                 | 1131.52                           | 0.87          | 2.5e-04       |             |                          |                                           |
|             |                       |                          | EITALAPSTMK Mox                     | 589.62                 | 1176.61                           | 0.62          | 1.8e01        |             |                          |                                           |
|             |                       |                          | AVFPSIVGRPR                         | 600.25                 | 1197.70                           | 0.80          | 4.2e-04       | 294          | 21                       | Structural constituent of cytoskeleton    |
|             |                       |                          | QYEDESGPSVHR                        | 758.97                 | 1515.70                           | 0.22          | 2.1e-03       |             |                          |                                           |
|             |                       |                          | MWHTFYNELR                          | 758.98                 | 1514.79                           | 1.21          | 1.1e-02       |             |                          |                                           |
|             |                       |                          | VAPEHPVLLTEAPLPK1                   | 977.89                 | 1953.06                           | 0.71          | 5.3e-06       |             |                          |                                           |
| 8           | Nucleophosmin          | P06748                   | WFEENASQSTVX                        | 713.86                 | 1424.66                           | 1.05          | 7.1e-04       | 131          | 7                        | Transcription factor                      |
|             |                       |                          | VKEPAPDETSFEALLK                    | 866.46                 | 1730.91                           | 0.01          | 1.5e-06       |             |                          |                                           |
| 9           | Fibrillarin            | P22087                   | DILNLA                              | 786.35                 | 785.46                            | -0.13         | 4.5e01        | 241          | 19                       | Ribonucleoprotein                         |
|             |                       |                          | IVLAHTFLR                           | 628.16                 | 1253.72                           | 0.58          | 2.8e-04       |             |                          |                                           |
|             |                       |                          | VISIEGDDKIEYR                       | 756.26                 | 1509.73                           | 0.77          | 5.7e-05       |             |                          |                                           |
|             |                       |                          | DHAIVWGVYRPPKK                      | 767.76                 | 1532.85                           | 0.67          | 5.0e-05       |             |                          |                                           |
|             |                       |                          | MQQENMKPQEOQTLTEPYER 2Mox           | 809.06                 | 2423.11                           | 1.05          | 7.0e-02       |             |                          |                                           |
| PCNA        |                       | P12004                   | FSASGELGNGNIK                       | 647.94                 | 1292.64                           | 1.23          | 8.7e-04       | 59           | 11                       | Regulation of cell cycle                  |
signaling in breast cancer cells. Together our data indicate that in the absence of Ku70, TrkA signaling is proapoptotic, and therefore this protein might be an important key to understanding the well known paradox of anti- versus pro-apoptotic activity of tyrosine kinase receptors. The data presented in Fig. 5C show the efficiency of RNA interference and cell transfection in decreasing the level of Ku70 and overexpressing TrkA, respectively.

DISCUSSION

The NGF-induced TrkA signaling pathway has mostly been studied in neuronal cells, and little is known about intracellular events occurring in breast cancer cells. The aim of the present work was to decipher the signaling initiated by TrkA in breast cells using a proteomics approach combining the co-immunoprecipitation of TrkA plus its putative intracellular partners and the identification of these proteins by mass spectrometry. Similar experimental protocols were used previously to elucidate epidermal growth factor (EGF), platelet-derived growth factor, or fibroblast growth factor signaling pathways (24–29), allowing the identification of new partners involved in tyrosine kinase receptor signaling. For example, it has been shown that Vav-2 is a substrate of the EGF and platelet-derived growth factor receptors (26), that NSAP1 and TOM1/1 are involved in the fibroblast growth factor signaling cascade (27), and that the valosin-containing protein (VCP) and actin are targeted by the serine/threonine kinase Akt (28, 29). In essence, the use of co-immunoprecipitation to study receptor signaling offers the advantage of enabling the detection of previously unsuspected signaling elements.

Some of the putative TrkA signaling partners that we identified here have already been described in growth factor-mediated pathways. This is for example the case for the Ras GTPase-activating-like protein IQGAP1 known as a scaffold protein that interacts with multiple components of signaling cascades (30). A mass spectrometry-based proteomics study combining SILAC (stable isotope labeling by amino acids in cell culture) and affinity purification showed that IQGAP1 is recruited by the activated Grb2-EGF receptor complex (31). Moreover a direct binding of IQGAP1 with the MAP kinase/ERK kinase (MEK) 1/2 and ERK2 has been demonstrated, suggesting that IQGAP1 is a scaffold for mitogen-activated protein kinase signaling. Because TrkA signaling involves activation of the MAP kinase cascade in breast cancer cells (9, 16) leading to the stimulation of cellular growth, IQGAP1 could also be a scaffold protein in this mitogenic pathway. Another protein already described in growth factor signaling was the VCP that belongs to the ATPases associated with various cellular activities (AAA) family and is involved in several ATP-dependent processes including ubiquitin-mediated proteolysis, DNA repair, apoptosis, or cell cycle control (32). Recently it was demonstrated that VCP is a target of Akt signaling (33) and is necessary for the antiapoptotic effect of Akt in breast cancer cells (28). The PI3K/Akt pathway is one of the cascades activated by TrkA in neuronal cells, and our present work suggests that VCP could also be involved in this pathway in breast cancer cells. In line with this, the cytoskeleton protein actin and its associated proteins have been shown to be targeted by Akt signaling (29, 34), leading to cytoskeleton reorganization, and the fact that we identified actin in our experiments corroborates other studies arguing in favor of the presence of actin in complex with TrkA and its involvement in...
TrkA signaling (18, 35). Another putative TrkA partner that we identified is nucleophosmin, also known as B23 or numatrin. Nucleophosmin is a protein implicated in cell growth and proliferation whose expression rapidly increases in response to mitogenic stimuli, and nucleophosmin was found at high levels in proliferative and malignant cells (36). Interestingly it has been shown recently that nucleophosmin mediates the antiapoptotic effect of NGF via the phosphatidylinositol 3-kinase enhancer/PI3K pathway (37, 38). Together with our present work, these data indicate that the role of nucleophosmin in the cytoprotective activities of NGF might be regulated by TrkA activation in breast cancer cells.

It should be noticed that co-immunoprecipitation and denaturing elution bring down a lot of immunoglobulins that may mask some real interaction partners while eluting some proteins that are not real TrkA partners. Therefore it is important to emphasize that new putative interaction partners identified in co-immunoprecipitation experiments must subsequently be validated by implementing a combination of approaches such as antibody recognition, confocal microscopy analysis, and functional validation by RNA interference. In our present study, this was the case for the protein Ku70, which was not known to be involved in TrkA signaling and for which we were able to go through the full validation process. Our results showed that Ku70 interacts with TrkA and is tyrosine phosphorylated upon NGF stimulation of breast cancer cells. The interaction of Ku70 with another growth factor receptor, the EGF receptor, has already been reported (39). This work demonstrated, by co-immunoprecipitation, that Ku70 can bind to the EGF receptor when it is inhibited with a blocking mono-

![Fig. 4](image-url)

**Fig. 4.** The protein Ku70 is tyrosine phosphorylated upon NGF stimulation in breast cancer cells. A, immunoprecipitation of tyrosine phosphorylated proteins (pY IP) in MCF-7 cells stimulated with 200 ng/ml NGF for 10 min revealed a 72-kDa differential band (indicated by an arrow) by Coomassie Blue staining that was subjected to trypsin digestion before mass spectrometry identification. Ab, antibodies used as an equal loading control. The activation of TrkA after NGF stimulation was checked by pY immunoprecipitation followed by immunoblotting (IB) with anti-TrkA. B, MALDI-TOF spectrum of the 72-kDa band observed in A. Peptides that matched with the theoretical tryptic peptides from human Ku70 are indicated. C, peptide mass fingerprint identification of human Ku70. Masses of the experimental charged tryptic peptides (MH+ submitted) and of their corresponding theoretical peptides (MH+ matched) are listed, and the peptide masses are indicated in Da. Sequences corresponding to each tryptic peptide are indicated with the position of the first and the last amino acid in parentheses. Peptides sequenced by tandem mass spectrometry are underlined. Mox indicates a methionine oxidation, and Pyro-glutamate (N-term Q) indicates a pyroglutamate modification of the N-terminal glutamine. D, immunoprecipitation of tyrosine phosphorylated proteins (pY IP) in PC12 and MCF-7 cells after NGF treatment (200 ng/ml for 10 min) followed by an immunoblot (IB) of Ku70 (AHP316 antibody) showed the tyrosine phosphorylation of Ku70 in NGF-stimulated MCF-7 cells. Ab, Ponceau detection of antibodies used as an equal loading control.
clonal antibody. The authors suggested a role of the EGF receptor in the sequestration of Ku70 in the cytoplasm thereby preventing its action in DNA repair as opposed to a direct role of Ku70 in the EGF signaling pathway. Although Ku70 is mainly localized in the nucleus, a cytosolic form of Ku70 has also been described (40–42). This is in agreement with our confocal microscopy study that showed a co-localization between TrkA and Ku70 in the cytoplasm of MCF-7 cells. In addition, we showed that Ku70 is phosphorylated on tyrosine as a consequence of TrkA activation by NGF. Although we were not able to map the tyrosine residues that are actually phosphorylated after NGF stimulation, potential sites of phosphorylation were identified on tyrosine 321 and 408 (data not shown) by in silico analysis using Scansite software (43). In addition, the possible role of Ku70 in the TrkA signaling pathway was investigated by using the inhibition of its expression by RNA interference. Our results showed that whereas depletion in Ku70 had no effect on its own on breast cancer cell survival and sensitivity to apoptosis, in contrast, in cells overexpressing TrkA, a decrease in Ku70 level resulted in a potentiation of apoptosis. Interestingly a previous study has reported that the cytosolic form of Ku70 binds to the proapoptotic signaling protein Bax and can inhibit Bax-mediated apoptosis by suppressing its mitochondrial relocalization from the cytosol (23), and it is tempting to propose that the hypersensitivity to TRAIL-induced apoptosis that we observed with the inhibition of Ku70 expression could be due to the decrease of Bax sequestration in the cytosol. Alternatively the DNA repair activities of Ku70 could also be at play here as this protein belongs to the DNA repair family of proteins and is a regulatory subunit of the DNA-dependent protein kinase. It is well established that DNA repair is necessary during cell proliferation and that its impairment can lead to cell death, but to date, no relationships between TrkA signaling and DNA repair have been described. Further targeted investigations would be necessary to determine whether TrkA stimulation impacts DNA repair. Although at this stage the mechanism of Ku70 involvement cannot be ascertained, our results showed that TrkA signaling in breast cancer cells involves Ku70 and that, in the absence of Ku70, TrkA signaling can potentiate apoptosis.

An old and still unresolved paradigm in growth factor signaling resides in the fact that common signaling pathways can lead to a different cellular effect depending on cell types and physiological environment. This is well illustrated with the pheochromocytoma PC12 cells in which EGF induces cell proliferation, whereas NGF stimulates cell differentiation based on similar signaling events and phosphorylation cascades (44, 45). Although the time course and kinetics of phosphorylation cascades, such as transient or sustained MAP kinase activation (46, 47), have been proposed to explain the differential biological effect observed with tyrosine kinase receptor stimulation, it still cannot be excluded that specific effectors would be differentially involved (48). Interestingly in our experiments, we did not detect any interaction between Ku70 and TrkA in PC12 cells, suggesting that Ku70 has no function in the stimulation of neuronal cell differentiation observed upon NGF stimulation. In breast cancer cells, we also identified the PCNA, which is a marker of proliferation specifically overexpressed during the S phase of the cell cycle. It has been demonstrated previously that NGF regulates cell cycle arrest and neuronal differentiation by inducing, among others things, the expression of p21cip1/waf1 (49) and a decrease of PCNA expression in PC12 cells. In contrast, NGF...
is antiapoptotic and mitogenic for breast cancer cells (8–10), and NGF stimulation results in an increase in PCNA expression. Together our data suggest that Ku70 is not involved in TrkA signaling that leads to the differentiation of PC12 cells but is more specifically implicated in cell growth and antiapoptotic NGF signaling in the context of breast cancer cells. Another illustration of the duality of tyrosine kinase receptor signaling is represented by their eventual ability to induce apoptosis. Although tyrosine kinase receptors have widely been described as stimulating cell survival, a proapoptotic effect of tyrosine kinase stimulation has long been described in a variety of cell types and physiological conditions (50–57).

Interestingly our RNA interference experiments also showed that, in the absence of Ku70, TrkA signaling enhances apoptosis of breast cancer cells. TrkA has long been known to generally have an ambivalent effect on cell survival (58), and a proapoptotic effect of TrkA signaling has already been reported (56, 57). In our experiments, TrkA potentiated TRAIL-induced apoptosis only in the absence of Ku70, and therefore Ku70 can prevent TrkA from inducing apoptosis in breast cancer cells. These data suggest that Ku70 might be a switch for the regulation of the apparently ambivalent effect of TrkA, and more generally tyrosine kinase receptors, between prevention and induction of apoptosis.

In conclusion, our work provides for the first time a list of putative partners (plus some contaminants) of TrkA signaling in breast cancer cells and reveals Ku70 as an important component of this pathway. The functional validation of the implication of these proteins in the control of breast cancer cell growth and survival will have to be further explored, as was done here in the case of Ku70, and their precise functions in the deregulation of multiprotein signaling complexes occurring in cancer cells will have to be defined. Previous studies have shown variable levels of TrkA expression in human breast tumors as well as a relationship between TrkA and estrogen receptor levels, resulting in a prognostic value associated to the expression of TrkA (11–13). Ku70 is increasingly regarded as crucially involved in carcinogenesis (59), and based on our present results, it could be postulated that targeting Ku70 in tumors overexpressing TrkA might be a relevant way to induce targeted apoptosis of cancer cells. This hypothesis nevertheless requires further in vitro and in vivo investigations, but it should be emphasized that Ku70, as well as the other proteins identified in this study, potentially represent new molecular targets whose interest for future therapeutic strategies against breast cancer will have to be investigated.

* This work was supported in part by grants from the Ligue Nationale Contre le Cancer (Equipe labellisée 2006), by the Region Nord-pas-de-Calais, by a French national Research Agency (ANR) RIB grant (to B. B. R.), by an ARCUS grant from the French Ministry of Foreign Affairs and the Rhône-Alpes Region (MIRA) (to B. B. R.), and by the Association pour la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by INSERM.

¶ Recipient of a fellowship from the French Ministry for Research and Education and the Association pour la Recherche sur le Cancer.

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