Experimental Cell Research 347 (2016) 114–122

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research article

Caspase-8 expression and its Src-dependent phosphorylation on Tyr380 promote cancer cell neoplastic transformation and resistance to anoikis

G. Fianco [a,b], C. Cenci [b,1], D. Barilà [a,b,*]

[a] Department of Biology, University of Rome "Tor Vergata", 00133 Rome, Italy
[b] Laboratory of Cell Signaling, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Fondazione Santa Lucia, 00179 Rome, Italy

ARTICLE INFO

Article history:
Received 12 April 2016
Received in revised form
13 July 2016
Accepted 14 July 2016
Available online 16 July 2016

Keywords:
Caspase-8
Src kinase
Tyrosine phosphorylation
Glioblastoma
Hepatocellular carcinoma
Hypoxia
Anoikis

ABSTRACT

Caspase-8 expression is lost in a small percentage of tumors suggesting that the retention of its functionality may positively contribute to tumor progression. Consistently, several non-apoptotic functions of Caspase-8 have been identified and Caspase-8 has been shown to modulate cell adhesion, migration and to promote tumor progression. We have previously identified the Src-dependent phosphorylation of Caspase-8 on Tyr380 as a molecular mechanism to downregulate the proapoptotic function of Caspase-8; this phosphorylation occurs in colon cancer and may promote cell migration in neuroblastoma cell lines. However, the occurrence of Caspase-8 phosphorylation on Tyr380 and its significance in different carcinoma cellular models, have not been clarified yet.

Here we show that Caspase-8 expression may promote cell transformation in glioblastoma and in hepatocarcinoma cell lines. In these systems Caspase-8 is phosphorylated on Tyr380 in a Src kinase dependent manner and this phosphorylation is required for transformation and it is enhanced by hypoxic conditions. Using a cancer cellular model characterized by Src constitutive activation engineered to express either Caspase-8-wt or Caspase-8-Y380F we could show that Caspase-8 expression and its phosphorylation on Tyr380, but not its enzymatic activity, promote in vitro cell transformation and resistance to anoikis. This work demonstrates a dual role for Caspase-8 in cancer, suggesting that Tyr380 phosphorylation may represent a molecular switch to hijack its activity from tumor suppressor to tumor promoter.

1. Background

Caspase-8 is an apical caspase essential for the transduction of the apoptotic cascade in response to death receptor induced apoptosis [20]. Consistently, Caspase-8 expression is ablated through several mechanisms in many different tumors, including neuroblastoma, primitive neuroectodermal tumors, medulloblastoma and small cell lung carcinoma [15,18,25,33,36]; these observations support the idea that its loss may promote tumorigenesis by impairing the apoptotic response [35]. Nevertheless, Caspase-8 expression is retained and even increased in some tumors such as glioblastoma and hepatocellular carcinoma [11,26]. Consistently, despite its well known role in apoptosis, Caspase-8 activation has been more recently identified also as part of signaling pathways that mediate cell proliferation, cell adhesion and cell migration [1,2,7,8,17,31,37,38], which may overall support tumor initiation and progression.

These observations point to a dual role of Caspase-8 in cancer and suggest the presence of molecular mechanisms that may modulate Caspase-8 function switching it from the classical apoptotic role to other signaling pathways.

Our previous work identified Caspase-8 phosphorylation on Tyr380 as a major mechanism to modulate Caspase-8 apoptotic activity. We could show that Src kinase may directly phosphorylate Caspase-8 on Tyr380 and demonstrated that this phosphorylation significantly downregulates Caspase-8 enzymatic activity and Fas-induced apoptosis [4]. Tyr380 lies in the linker region that separates the p18 and the p10 subunit of Procaspase-8 and that is removed upon autocleavage at two aspartic residues, D374 and D384. This event is required for the consequent assembly of the tetrameric Caspase-8 complex constituted by two p18 and two p10 subunits. We could show that Tyr380 phosphorylation impinges on full Caspase-8 activation as it impairs the autoprocessing cleavage at these aspartic residues [4]. Our finding has been further supported by structural data on Procaspase-8 and by NMR

* Corresponding author at: Department of Biology, University of Rome "Tor Vergata", 00133 Rome, Italy.
E-mail address: daniela.barila@uniroma2.it (D. Barilà).
[1] Istituto Pasteur-Fondazione Cenci Bolognetti, Rome, Italy.

http://dx.doi.org/10.1016/j.yexcr.2016.07.013
0014-4827/

studies which clearly show that phosphorylation at Tyr380 significantly reduces the rate of the cleavage reaction [21,22]. Overall, Src-dependent phosphorylation of Caspase-8 on Tyr380 provides a molecular mechanism to downregulate the proapoptotic activity of Caspase-8 and it may promote the switch of its function from proapoptotic to tumorigenic. Consistently with this hypothesis, we identified endogenous Caspase-8 phosphorylation in colon cancer where Src activity is often aberrantly induced [4], and evidence for a role of Tyr380 phosphorylation in the promotion of cell adhesion, migration and tumor progression has been provided [1,31]. Interestingly, phosphorylated Tyr380 is a Src homology domain 2 (SH2) binding site and may therefore trigger the recruitment of several proteins and the subsequent induction of cell migration [31]. As an example, the interaction of phosphorylated Caspase-8 with the regulatory subunit p85alfa of PI3K (phosphatidylinositol-3-kinase) enhances Rac activation to promote cell migration [37,38]. Furthermore, Tyr380 of Caspase-8 is required for the assembly of the FAK-Caspase-8-Calpain complex where Caspase-8 promotes Calpain activity therefore enhancing migration and metastasis [2]. Although these findings support the idea that the presence of Caspase-8 expression, at least in some contexts, may represent a significant advantage for tumor growth, the definition of the tumorigenic contexts and the significance of Src dependent phosphorylation of Tyr380, are still largely obscure.

Here we provide evidence for the retention of Caspase-8 expression and of its Src-dependent phosphorylation on Tyr380 in glioblastoma and hepatocellular carcinoma cell lines. We demonstrate that this phosphorylation is enhanced in hypoxic conditions and that it contributes to neoplastic transformation and to the evasion of anoikis.

2. Methods

2.1. Cell culture

U87 MG (U87), U251 MG (U251) and HepG2 cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum.

Cell lines stably interfered for Caspase-8 were obtained by retroviral-mediated expression of short hairpin RNA (shRNA) using pRETRO Super Vector or pLV-cDNA lentiviral vector. Retroviruses and Lentiviruses were produced in Hek293T as previously described [34]. After 48 h from infection, stable polyclonal populations of control and Caspase-8 depleted cells were obtained by selection for two weeks with 2 μg/ml puromycin (Sigma Aldrich). The sequences of Caspase-8 used for interference are: shC8 5′-ATCACAGACTTTGGACAAA-3′, sh#2 5′-GCCTGGATGTTATTCCAG-3′ and shR5 as control 5′-GGATATCCCTCTAGATTA-3′.

U87 cell lines stably overexpressing SrcK295R were obtained by retroviral infection using the pLNCX-SrcK295R vector, a gift from Joan Brugge (Addgene plasmid # 13659).

Colorectal tumor cell lines, DLD-1, DLD-1-TR, DLD-1-TR-pSMV, DLD-1-TR-C8 and DLD-1-TR-C8YF, were maintained in RPMI medium supplemented with 10% fetal bovine serum. To generate DLD-1-TR-C8 and DLD-1-TR-C8YF cell lines, the coding sequence of Caspase-8 wt and Caspase-8 Y380F were cloned in pBILuc vector. This vector was cut in sites AseI, as well as pN1pβActin-rtTA2S-M2-IRES-EGFP vector. Fragment of Caspase-8 in pBILuc that contains Caspase-8 (wt and Y380F) was inserted in pN1pβActin-rtTA2S-M2-IRES-EGFP vector to create the Super Module Vector (pSMV). Cells were transfected with the above vectors or with empty pSMV as control and selected with Neomycin (G418; Sigma-Aldrich) at the final concentration of 500 μg/ml for 30 days [39].

Stably reconstituted cell lines (DLD-1-TR-pSMV, DLD-1-TR-C8 and DLD-1-TR-C8YF) were supplemented with Neomycin G418 100 μg/ml for selection. The induction of Caspase-8 expression (wt and Y380F) was achieved upon Doxycycline (Sigma-Aldrich) treatment for the indicated times at the final concentration of 2 μg/ml.

2.2. Immunoprecipitation and immunoblot analysis

Cell extracts were prepared in IP buffer (50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml TPCK, 5 μg/ml TLCK, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin). For immunoblotting, 50–100 μg of protein extract were separated by SDS–PAGE and blotted onto nitrocellulose or PDVF membrane; the proteins of interest were detected with specific antibodies. For immunoprecipitation, protein extracts prepared as above were incubated for 1–2 h with specific antibody *anti* Caspase-8 previously conjugated to proteinA-sepharose (GE-Healthcare). Immune complexes were then resolved and analysed by SDS–PAGE. All immunoblots were revealed by ECL (GE Healthcare). The following antibodies have been used for Western Blot analysis: *anti* Caspase-8 (MBL 1:1000); *anti* phospho Y380 Caspase-8 (E11, EMBL 1:500); *anti* Src (Calbiochem 1:500); *anti* phospho-Y418 Src (Invitrogen 1:500); anti Tubulin (Sigma-Aldrich 1:2000); anti Rac (Millipore 1:500); anti PARP (Cell Signaling 1:1000) HIF-1α (BD 1:1000); anti-pTyr 4G10 (Millipore 1:500).

2.3. Rac-activity assay

Protein extracts were obtained using a specific buffer solution (50 mM TRIS pH 7.5, 100 mM NaCl, 10% glycerol, 1% NP-40, 2 mM NaF, 100 mM Sodium Orthovanadate, 10 μg/ml cocktail and incubated for 90 min with the fusion protein GST-PAK, previously bounded to glutathione-sepharose (GE-Healthcare), on a rotating shaker at 4 °C.

2.4. Soft agar assay

Anchorage-independent growth assays were performed as described [10]. Data on biological assays are representative of three independent experiments performed in triplicate. For each point 5×10^3 cells (DLD1, DLD1-TR, DLD-1-TR-C8 and DLD-1-TR-C8YF cell lines) and 1×10^4 cells (U87, U251, HepG2 cell lines, silenced or not for Caspase-8 expression) were used.

2.5. Anoikis assay and analysis of cell death

DLD-1, DLD-1-TR and reconstituted cells, were plated ($3{,}2 \times 10^5$) in six wells plated pre-treated with 12 mg/ml poly-HEMA Sigma Aldrich diluted in 95% Ethanol Sigma Aldrich to prevent cell adhesion to the substrate and induce anoikis. Cells were grown in absence of anchorage for 16 h. Cell death was determined by flow-cytometry analysis of DNA fragmentation upon propidium iodide (100 μg/ml in PBS) nuclear staining. Specific cell death was determined as follow: (% of apoptotic cells in suspension - % of apoptotic cells in adhesion)/100 - % of apoptotic cells in adhesion). Alternatively, cell death was evaluated by trypan blue incorporation.

2.6. Cell proliferation assay

DLD1, DLD1-TR, TRAIL R pSMV Caspase-8 wt or Y380F cells (Caspase-8 expression was induced or not by Doxycycline) were maintained for 72 h in DMEM supplemented with 10% FBS. All cell lines were seeded in triplicate in six-well plates at density of 4×10^4 cells per well in the same medium. Data on biological