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The transcription factor STAT3 is increasingly viewed as an oncogene. Activated STAT3 is pro-proliferative, anti-apoptotic and oncogenic. There is growing evidence for the epigenetic regulation of STAT3 activity in cancer cells. We and others have previously reported that STAT3 activity in breast cancer and in lung endothelial and epithelial cells was inversely regulated by the tumor suppressor protein caveolin-1 (cav-1). In order to understand the mechanisms involved, we reexamined how STAT3 signaling, which is initiated in plasma membrane raft microdomains, traverses the cytoplasm to the nucleus. Using cell-fractionation methods, we observed that by 15-30 min. after IL-6 treatment, up to two thirds of cytoplasmic Tyr-phosphorylated STAT3 can be associated with the purified early endosome (EE) fraction (Rab-5-, EEA1-, TfR- and clathrin-positive fraction). Electron microscopy, immunofluorescence and detergent-dissection approaches confirmed the association of STAT3 and PY-STAT3 with early endosomes. STAT3-transcriptional activation was inhibited by expression constructs for dominant negative dynamin K44A, epsin 2a, ampiphysin A1 and clathrin light chain, but enhanced by that for the active dynamin species MxA. Moreover, overexpression of the tumour suppressor cav-1 negatively regulated STAT3 signaling. Taken together, the data demonstrate strong epigenetic regulation of STAT3 signaling in cancer cells by virtue of dynamic membrane-associated trafficking along the caveolar (negative regulation) and endocytic (positive regulation) pathways.

Key words: STAT3, caveolin-1, oncogene, tumor suppressor, transcriptional regulation, signaling endosomes.

Epigenetic regulation of transcription factor STAT3 activity in cancer cells

Epigenetyczna regulacja aktywności czynnika transkrypcyjnego STAT3 w komórkach nowotworowych

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Introduction

The transcription factor STAT3 has been characterized as an oncogene in the context of breast cancer [1, 2]. Activated STAT3 is pro-proliferative, anti-apoptotic and oncogenic [1, 2]. Several studies to investigate the relationship between nuclear STAT3 and phospho-STAT3 with breast cancer prognosis are currently ongoing [1-3]. The increased incidence of breast cancer with advancing age has been very recently linked to the increased secretion by breast stromal fibroblastic cells of interleukin-6 (IL-6)[4], which, in turn, activates STAT3 [5]. Conversely, inhibition of gp130 receptor signaling (the pathway used by IL-6-type cytokines) has been shown to reduce this "constitutive" activation of STAT3 in breast cancer cells and to reduce in vivo malignancy [6]. Moreover, mutations in the membrane raft and "tumor suppressor" protein caveolin-1 (cav-1) are common in human breast cancer (16% of cases) [reviewed in 7]. Experimentally, cav-1 loss (by genetic knockout) in the mouse leads to hyperactivation of STAT3 and STAT5, increased incidence of breast cancer and increased metastases of breast cancer to the lungs [7, 8]. STAT3 is also the major transcriptional factor activated in target cells by various gene therapy protocols which use the IL-6-type family of cytokines and their receptors. In this review we discuss a novel aspect of STAT3 function: the epigenetic regulation of its transcriptional activity by the tumor suppressor protein cav-1 and the trafficking of activated STAT3 through the cytoplasm along the membrane-associated endocytic pathway [9-11].

IL-6 and STAT3 in breast cancer

In 1989 we were the first, using immunohistochemistry, to report the presence of IL-6 in neoplastic cell elements and stromal tissue of primary carcinomas of mammary, colonic, ovarian and endometrial origin as well as in adenocarcinomatous metastases to lymph nodes [12]. Additionally, we observed constitutive IL-6 immuno-staining in breast cancer cell lines (e.g. T47D cells) [12]. In vivo, IL-6 was an invariant presence in the host-tumor interaction, and is thought to contribute to symptoms of the paraneoplastic syndrome and cancer cachexia [reviewed in 13, 14].

In a line of research begun in the early 1990s, we observed that IL-6 increased breast ductal epithelial cell dyshesion and cell motility (in T47D and ZR-75-1 cells) [15-17]. Subcellular events accompanying this epithelioid to mesenchymal transformation included an IL-6-induced loss of focal adhesions and reduction in E-cadherin [15-18]. Other investigators also now relate IL-6, in cooperation with EGF, and scatter factor and TGF-α, to increased dyshesion and migration of breast cancer cells [reviewed in 17, 19, 20]. The increased incidence of breast cancer with advancing age has been very recently linked by Campisi et al. [4] to the increased secretion by breast

Czynnik transkrypcyjny STAT3 jest w coraz większym stopniu określany mianem onkogenu. Aktywowany STAT3 zwiększa proliferację komórek oraz ma działanie antyapoptotyczne i onkogenne. Coraz więcej dowodów wskazuje na epigenetyczną regulację STAT3 w komórkach nowotworowych. Poprzednio donoszono, że aktywność STAT3 w komórkach raka piersi oraz w komórkach śródbłonkowych i nabłonkowych płuc jest hamowana przez białko supresorowe kaweolinę-1 (cav-1). W celu zrozumienia mechanizmów zaangażowanych w ten proces regulacji ponownie zbadano, jak odbywa się przekazywanie sygnału za pośrednictwem STAT3 z błony komórkowej poprzez cytoplazmę do jądra. Używając metod frakcjonowania komórkowego zaobserwowano, że przez 15–30 min po poddaniu komórek działaniu interleukiny-6 (IL-6) nawet 2/3 fosforylowanego STAT3 może być związane z oczyszczoną wczesną frakcją endosomalną (EE – frakcja zawierająca Rab-5, EEA1, TfR i klatrynę). Mikroskopia elektronowa, immunofluorescencja i rozdzielanie w detergencie potwierdziły istnienie związania STAT3 i jego fosforylowanej postaci z wczesnymi endosomami. Aktywacja transkrypcji przez STAT3 była hamowana przy pomocy konstruktów ekspresyjnych wytwarzających negatywnie dominującą formę dynaminy K44A, epsynę 2a, ampifizynę A1 i lekki łańcuch klatryny, ale ulegała wzmocnieniu pod wpływem aktywnych postaci dynaminy MxA. Co więcej, nadekspresja supresora cav-1 negatywnie regulowała przekazywanie sygnału przez STAT3. Powyższe dane wskazują na silną epigenetyczną regulację przekazywania sygnału przez STAT3 w komórkach nowotworowych. Regulacja ta odbywa się zarówno poprzez wpływ na szlak kaweolarny (regulacja negatywna), jak i szlak endocytarny (regulacja pozytywna).

Słowa kluczowe: STAT3, kaweolina-1, onkogen, supresor guza, regulacja transkrypcji, endosomy sygnałowe.

stromal fibroblastic cells of interleukin-6 (IL-6). Conversely, inhibition of gp130 receptor signaling (the pathway used by IL-6-type cytokines) has been shown to reduce this "constitutive" activation of STAT3 in breast cancer cells and to reduce in vivo malignancy [6]. The importance of STAT3 activation in breast cancer cells is further highlighted by (a) the recognition that scatter factor, a cytokine long investigated for its ability to promote breast cancer cell motility and "scattering" [17], is also now known to signal via the c-met receptor/STAT3 pathway [21], (b) that other growth factors and cytokines relevant to breast cancer invasiveness and metastasis (e.g. EGF, PDGF) also activate STAT3 signaling [5], (c) the identification of specific genes upregulated by STAT3 which contribute to enhanced breast cancer invasiveness (e.g. matrix metalloproteinase 9, MMP9) [1], and (d) the demonstration that small molecule inhibitors of activated STAT3 such as indirubin and STA-21 reduce the proliferative phenotype of breast cancer cells [22, 23].

Initiation of cytokine/STAT3 signaling in plasma membrane raft microdomains

Diverse cytokines and growth factors, including IL-6, EGF, PDGF, scatter factor, activate the JAK/STAT3 signaling pathway leading to Tyr-phosphorylation (by JAKs) and Ser-phosphorylation (via the ras-raf-MAPK pathway) of STAT3 [5]. We reported in 2002 that STAT3 activation at the plasma membrane took place in specialized microdomains called "rafts" (the "STATs in rafts" hypothesis; 24) in complexes with the raft protein cav-1 and the chaperones HSP90 and GRP58 [25, 26]. We have shown earlier that a consequence of reduced cav-1 in rafts is the hyperactivation of STAT3 signaling and, conversely, that overexpression of wt cav-1 inhibits STAT3 signaling in tumor cells (27, 28). Lisanti and colleagues have also shown hyperactivation of STAT5 in breast tissue of cav-1 KO mice together with breast epithelial cell hyperplasia [7, 29]. Moreover, breast tumor cells showed increased invasiveness and lung metastasis in cav-1 KO mice [29]. In additional studies, hyperactivation of STAT3 in different tissues has been confirmed in cav-1 KO mice [8]. Breast cancer cells expressing exogenously introduced wt cav-1 showed reduced tumorigenesis and invasiveness [29].

Epigenetic regulation of STAT3 transcriptional activity along the endocytic pathway

Since 1999, research in this laboratory has resulted in novel insights into our understanding of how STAT3 works. Specifically, our previous position that latent STAT proteins do not exist in the cytosol as free monomers as per the original Darnell model of 1994 [reviewed in 5] but in large complexes [9, 30, 31] is now widely accepted, including by the Darnell group [32, 33]. In recent years we [11] and Bild et al. [10] have discovered a novel aspect of STAT3 trafficking and activation in the cytoplasm: the majority (approximately 75%) of cytoplasmic trafficking of PY-STAT3 is membrane associated (Fig. 1). Using cell-fractionation methods, we observed that approximately 5% of cytoplasmic STAT3 was constitutively associated with the purified early endosome (EE) fraction in human hepatoma Hep3B cells (which have low endogenous cav-1). By 15-30 min. after IL-6 treatment, up to two thirds of cytoplasmic Tyr-phosphorylated STAT3 can be associated with the purified early endosome fraction (Rab-5-, EEA1-, TfR- and clathrin-positive fraction). Electron microscopy, immunofluorescence and detergent-dissection approaches confirmed the association of STAT3 and PY-STAT3 with early endosomes. Further immunofluorescence and cell fractionation studies confirmed the association of STAT3 with the Rab7 and LAMP1, 2-positive late endosomal/lysosomal compartment. Biochemically, STAT3 was constitutively in complexes with clathrin heavy chain (CHC) in membrane and cytosolic compartments. As with clathrin, the membrane association of STAT3 was dynamic in that within 15 min. of treatment with the vicinal-thiol crosslinker phenylarsine oxide (PAO) there was a dramatic increase in bulk STAT3

association with sedimentable membranes. The functional contribution of PY-STAT3 association with the endocytic pathway was evaluated in transient transfection assays using IL-6-inducible STAT3-reporter-luciferase constructs and selective regulators of this pathway. STAT3-transcriptional activation was inhibited by expression constructs for dominant negative dynamin K44A, epsin 2a, ampiphysin A1 and clathrin light chain, but enhanced by that for the active dynamin species MxA. Taken together, the new data [11 and citations therein concerning the negative STAT3 regulators hepatocyte growth factor receptor tyrosine kinase substrate HRS and schwanomin] demonstrate strong epigenetic regulation of STAT3 signaling by virtue of dynamic membrane-associated trafficking along the caveolar (negative regulation) and endocytic (positive regulation) pathways. In other words, STAT3 dynamics at the plasma membrane and in the cytoplasm are inextricably linked to those of caveolin, clathrin, endocytosis and the biochemical regulatory machineries involved at intracellular membrane surfaces (the "STAT3 signaling endosome hypothesis"; Fig. 1).

The "STAT3 signaling endosome hypothesis": a platform for cross-talk with other pathways

As a template for understanding where our studies fit into the broader signaling literature, the convergence of caveolar and endocytic pathways in the regulation of cellular signaling is becoming apparent in several other experimental systems. Available data show convincingly the merging of caveolar and endosomal pathways [34-36]. With respect to ligand-activated signaling, the formulation of a "signaling endosome hypothesis" in the mid 1990s with respect to NGF action has drawn attention to the ability of clathrin-coated endosomal vesicles to mediate rapid and directional transit of signaling molecules through the cytoplasm in [37-39]. In PC12 cells, NGF recruited CHC and TrkA into endosomal membrane-associated complexes with AP2 adaptor which then served as a platform for the ras-MAPK pathway [37-39]. Moreover, this endomembrane-associated signaling pathway was bifurcated in that of the two NGF receptors p75NTR was eventually partitioned towards recycling endosomes while TrkA was directed towards late endosomes/lysosomes for degradation [39]. Additional evidence for a functional bifurcation during internalization comes from the demonstration that ligand-induced raft/TGF-βR/Smad transcriptional signaling was regulated negatively by cav-1 as a result of targeting to lysosomes and regulated positively by targeting to endosomes [40]. The complexities of dual positive and negative signaling during vesicular internalization have been further highlighted by the observation that clathrin-dependent and clathrin-independent pathways can be differentially engaged in the case of EGF signaling based upon ligand concentration and ubiquitination of epsin/AP2 adaptors [41, 42].

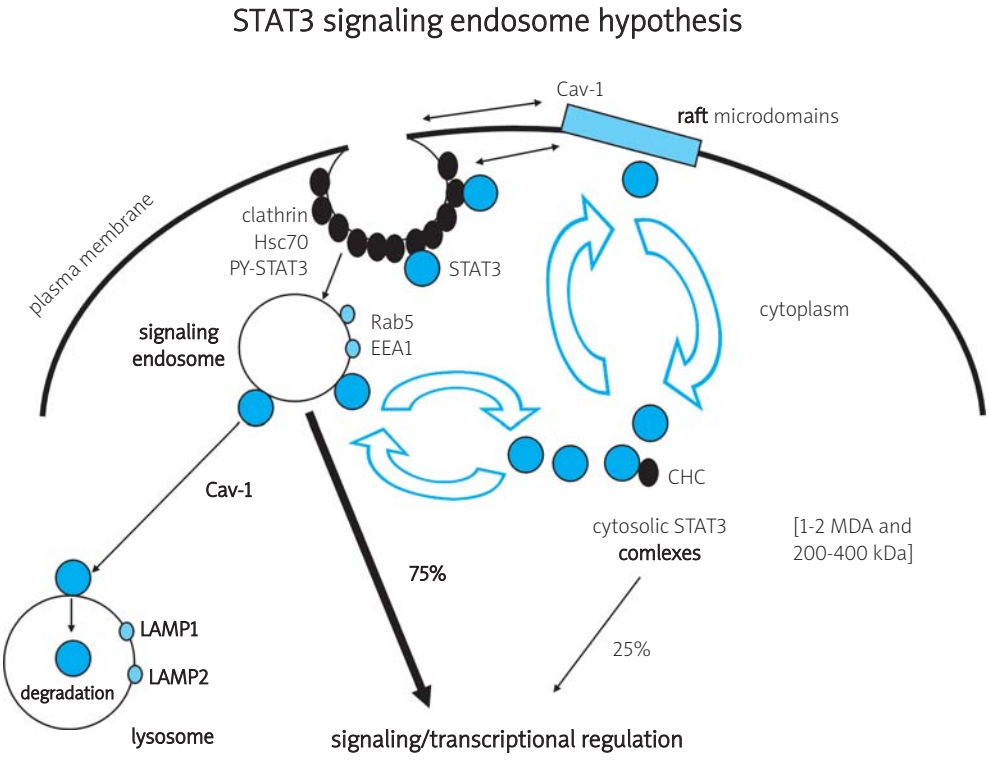

Fig. 1. Epigenetic regulation of the transcriptional activity of STAT3: regulation by caveolin-1 and clathrin
Ryc. 1. *Epigenetyczna regulacja aktywności STAT3 jako czynnika transkrypcyjnego: regulacja przez kaweolinę-1 I klatrynę*

In the case of IL-6/gp130/STAT3 signaling, it is clear that there is constitutive internalization of gp130 in interaction with the AP2 adaptor complex [43]. Moreover, Bild and colleagues have shown that EGF- and PDGF-activated STAT3 co-localize in the cytoplasm with vesicular elements which also contain the α-adaptin polypeptide of the AP2 complex [10]. More generally, receptors for diverse cytokines which activate STAT signaling are known to traffic along the endocytic pathway in a manner dependent upon their Leu-Leu or Leu-Ile internalization motif [44]. Recently Meads and Medveczky [45] have interpreted their findings on the occurrence of gp130/STAT3 signaling, as assayed in terms of Tyr-phosphorylation of cytoplasmic STAT3, by vIL-6 mutants which do not appear to be secreted, as activation from within the endoplasmic reticulum/Golgi compartments. Similar observations of activation of the JAK/STAT pathway by secretion defective truncated interferons α, β or γ apparently from within the cell have also been reported [46 and citations therein]. Our hypothesis concerning the constitutive and dynamic association of STATs with cytoplasmic vesicular elements provides a basis for understanding these observations.

Is there a kinetic advantage of the endosomal signaling pathway compared to a cytosolic random walk mechanism? Following up on the original proposal of a "signaling endosome" with NGF, Howe [47] carried out computer modeling studies of PY-STAT3 traverse from the plasma membrane to the nucleus using a non-directional random walk or a directional endosomal trafficking paradigm. Even in 2-D simulations using conservative assumptions of a Stokes radius for STAT3 equivalent to 100 kDa, cellular geometry and cytosolic viscosity, directional endosomal trafficking reached the nucleus from the plasma membrane in 1 sec., but the random walk from the plasma membrane failed to do so. Thus, directional endosomal trafficking was kinetically more efficient than a random walk through the free cytosol [47].

Conclusions

The space between the plasma membrane and the cell nucleus is not "empty" as is depicted in innumerable schemes describing signal transduction initiated by cytokines and growth factors. The trans-cytoplasmic traverse of an activation signaling appears to be efficiently mediated in association with the cytoplasmic membrane pathways – the endocytic pathway – which exerts both positive and negative influence on the eventual nuclear targeting and function of the respective activated transcription factors such as STAT3.

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