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研究成果の刊行に関する一覧表

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雑誌

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植田 弘師	ネクローシスを抑制する脳保護タンパク質プロサイモシンα	***日薬理誌***	135	219	2010
Ueda H, Matsunaga H, Uchida H, Ueda M	Prothymosin alpha as robustness molecule against ischemic stress to brain and retina.	***Ann N Y Acad Sci***	1194	20-26	2010
Matsunaga H, **Ueda H**	Stress-induced non-vesicular release of prothymosin-alpha initiated by an interaction with S100A13, and its blockade by caspase-3 cleavage.	***Cell Death Differ***	17	1760-1772	2010
Ueda H	Prothymosin alpha and cell death mode switch, a novel target for the prevention of cerebral ischemia-induced damage.	***Pharmacol Ther***	123	323-333	2009
Fuhita R, Ueda M, Fujikawa K, **Ueda H.**	Prothymosin-alpha plays a defensive role in role in retinal ischemia through necrosis and apoptosis inhibition.	***Cell Death Differ***	16	349-358	2009

S3C33-1 Anti-high mobility group box1 monoclonal antibody ameliorates brain infarction

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High mobility group box1 (HMGB1), originally identified as an architectural nuclear protein, exhibits an inflammatory cytokine-like activity in the extracellular space. Here we show that treatment with anti-HMGB1 monoclonal antibody (mAb) remarkably ameliorated brain infarction induced by 2-h occlusion of the middle cerebral artery in rats, even when the mAb was administered after the start of reperfusion. Consistent with the 90% reduction in infarct size, the accompanying neurological deficits in locomotor function were significantly improved. mAb inhibited the increased permeability of the activation of microglia, the expression of TNF-alpha and iNOS, and suppressed activity of MMP-9. Immunohistochemical study revealed that HMGB1 immunoreactivity in the cell nuclei decreased or disappeared in affected areas, suggesting the release of HMGB1 into the extracellular space. Anti-HMGB1 mAb efficiently inhibited the development of brain edema through the protection of blood-brain barrier (BBB) structure in the early reperfusion phase following focal ischemia of brain tissue. These results indicate that anti-HMGB1 mAb inhibits the development of brain infarction through the protection of BBB structure in the ischemic region.

S3C33-2 Therapeutic potential of non-psychotropic cannabidiol in ischemic stroke

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Cannabis contains the psychoactive component delta9-tetrahydrocannabinol (delta9-THC), and the non-psychoactive components cannabidiol (CBD), cannabinol, and cannabigerol. It is well-known that delta9-THC and other cannabinoid CB1 receptor agonists are neuroprotective during global and focal ischemic injury. Additionally, delta9-THC also mediates psychological effects through the activation of the CB1 receptor in the central nervous system. In addition to the CB1 receptor agonists, cannabis also contains therapeutically active components which are CB1 receptor independent. Of the CB1 receptor-independent cannabis, the most important is CBD. In the past five years, an increasing number of publications have focused on the discovery of the anti-inflammatory, anti-oxidant, and neuroprotective effects of CBD. In particular, CBD exerts positive pharmacological effects in ischemic stroke. The cerebroprotective action of CBD is CB1 receptor-independent, long-lasting, and has potent anti-oxidant activity. Importantly, CBD use does not lead to tolerance. Among cannabis compounds, CBD may represent a very promising agent with the highest prospect for therapeutic use for ischemic stroke.

S3C33-3 Prothymosin α: a novel neuroprotetive polypeptide against ischemic damages

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In stroke, both necrotic and apoptotic neuronal cell death cause the loss of functions that include memory, sensory perception and motor skills. Since necrosis potentially expands cell death, while apoptosis restricts the spread of irretrievable damage, neuronal necrosis is considered to be a principle target for the rapid treatment of stroke. Prothymosin α (ProTα), a highly acidic nuclear protein that lacks signal peptide, was isolated from the conditioned medium after serum-free culture of cortical neurons and found to convert a cell death mode switch from necrosis to apoptosis. Indeed, ProTα administered via systemic routes markedly inhibited the functional and histological damages induced by cerebral and retinal ischemia. Although ProTα converted a cell death mode switch from necrosis to apoptosis *in vivo*, the ProTα-induced apoptosis was found to be completely inhibited by brain-derived neurotrophic factor or erythropoietin produced in the ischemic brain. Analysis in terms of the therapeutic time window and potency suggest that ProTα could be the prototypic compound to develop the medicine, and ProTα signaling may also be an important novel therapeutic strategy useful for the treatment of stroke.

S3C33-4 Understanding the mechanisms of actions of neuroprotective compounds in animal stroke models

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Stroke is the third leading cause of death in Japan accounting for approximately 10% of all deaths. Intravenous t-PA has been approved for treating acute ischemic stroke, but delayed treatment is associated with increased risk of cerebral haemorrhagic transformation. In light of this background, there is a need for novel drugs for treating stroke. We investigated the effects of two new compounds, which have nuroprotective actions. In this symposium, we will share with you their mechanisms of actions, which we have investigated in animal stroke models. One of the two compounds is GIF-0173, a derivative of prostaglandin J2. GIF-0173 reduced the infarct size by 40% in a cerebral thrombosis model. The neuroprotection was via activation of prostaglandin D1 receptor, which upregulates the sarcoplasmic/endoplasmic reticulum calcium ATPase pump activity in endoplasmic reticulum leading to reduction of free cytoplasmic calcium. Another compound we investigated is Ginkgolide B (GB). The reduction of infarct size by GB was 34% in animal stroke models. It protected neurons by reducing intracellular calcium influx through an NMDA receptor during excitotoxicity. The two compounds are potential candidates for the treatment of stroke.

最近の話題

日薬理誌 135, 219 (2010)

ネクローシスを抑制する脳保護タンパク質プロサイモシンα

植田 弘師

脳梗塞性脳卒中の（超）急性期には，コアとよばれる梗塞中心部の神経はネクローシス性の細胞死に陥り，神経細胞内からの細胞毒性物質の放出により二次的ネクローシスが誘発される．アポトーシス性の遅延性細胞死が観察されるコア周辺領域（ペナンブラ）が治療標的とされるが，最近ではアポトーシス抑制薬にはあまり顕著な効果が認められないこともあり，血栓溶解剤を用いた（超）急性期治療が重要視されている．アポトーシスに比べてネクローシスのメカニズムに関する研究はあまり多くないため，神経細胞死を標的とした治療には余り注目されてこなかったが，筆者らは培養神経細胞上清からネクローシス抑制タンパク質プロサイモシンαを発見し(1)，このタンパク質が脳梗塞性脳卒中モデルにおいて著効を示すことを見出したので，関連する最近の話題とともに紹介する(2,3)．このタンパク質は細胞核に局在する酸性のタンパク質であるが，ネクローシスを誘発させる無血清飢餓あるいは虚血性ストレス性の細胞ストレス時に細胞質局在を経ずに神経細胞から直接放出される．このようなストレス時には神経細胞膜に局在するグルコーストランスポーター1/4が急速に内在化し，続いてATP酸性低下によるエネルギー危機とそれに続くネクローシスが誘発される．プロサイモシンαを虚血ストレス前に添加すると，$G_{i/o}$タンパク質受容体，ホスホリパーゼC，プロテインキナーゼCの活性化を介してこのトランスポーター内在化が抑制され，ストレス後添加においても一旦内在化したトランスポーターの再膜局在化，結果としてネクローシス抑制が誘発される．プロサイモシンαは同様な機構を介して，培養条件下では遅延性のアポトーシス様の細胞応答を誘発するが，その意義はカスパーゼ3の活性化によるポリADPリボースポリメラーゼ分解がATP消費抑制につながり結果としてネクローシス抑制に働き，ネクローシス誘発までの時間稼ぎをすることであろうと考えられる（図）．培養条件下ではアポトーシス抑制性の神経栄養因子を添加することで，細胞死は完全に抑制されることから，プロサイモシンαは制御不能なネクローシス性細胞死から制御可能なアポトーシスにモード変換させる分子であるとも言えるであろう．In vivoでは虚血時に発現増加するアポトーシス抑制性の神経栄養因子の働きがあるので，プロサイモシンαの全身投与により脳虚血によるネクローシスとアポトーシス性の神経細胞死のいずれもが抑制され，強力な虚血脳保護作用が観察された．その活性は顕著であり，中大脳動脈の結紮後3時間の1回全身投与により脳組織障害や運動障害がほぼ完全に抑制されたという結果が得られている(4)．培養細胞で観察されるような細胞死モード変換と神経栄養因子によるアポトーシス抑制現象は網膜虚血時にも観察され，硝子体内へのBDNF抗体投与によりアポトーシス増加が認められている(5)．またこのモデルにおいてプロサイモシンα抗体やアンチセンス投与により，網膜虚血障害悪化が観察されることから，プロサイモシンαは虚血時の内在性神経保護分子であることが明らかとなった(5)．

著者らはプロサイモシンαのストレス性非古典的細胞外放出機構や受容体機構などにも手がかりを得ていることから，創薬という視点からさらなる研究発展を計画中である．

文　献

1) Ueda H, et al. J Cell Biol. 2007;176:853-862.
2) Ueda H. Pharmacol Ther. 2009;123:323-333.
3) 植田弘師. Medical Bio. 2008;5:83-89.
4) Fujita R, et al. Cell Death Differ. 2007;14:1839-1842.
5) Fujita R, et al. Cell Death Differ. 2009;16:349-358.

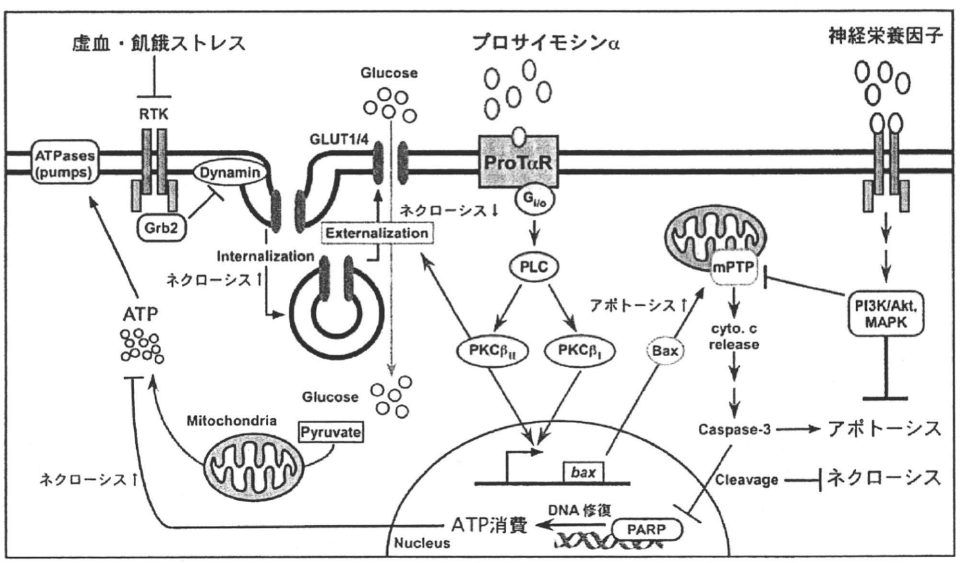

図　プロサイモシンαの神経細胞死保護機構

キーワード：プロサイモシンα，ネクローシス，虚血
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Prothymosin α as robustness molecule against ischemic stress to brain and retina

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Following stroke or traumatic damage, neuronal death via both necrosis and apoptosis causes loss of functions, including memory, sensory perception, and motor skills. As necrosis has the nature to expand, while apoptosis stops the cell death cascade in the brain, necrosis is considered to be a promising target for rapid treatment for stroke. We identified the nuclear protein, prothymosin alpha (ProTα) from the conditioned medium of serum-free culture of cortical neurons as a key protein-inhibiting necrosis. In the culture of cortical neurons in the serum-free condition without any supplements, ProTα inhibited the necrosis, but caused apoptosis. In the ischemic brain or retina, ProTα showed a potent inhibition of both necrosis and apoptosis. By use of anti-brain-derived neurotrophic factor or anti-erythropoietin IgG, we found that ProTα inhibits necrosis, but causes apoptosis, which is in turn inhibited by ProTα-induced neurotrophins under the condition of ischemia. From the experiment using anti-ProTα IgG or antisense oligonucleotide for ProTα, it was revealed that ProTα has a pathophysiological role in protecting neurons in stroke.

Keywords: apoptosis; brain-derived neurotrophic factor; cell death mode switch; ischemia; necrosis; robustness

Introduction

Stroke is a major cause of death and a major factor behind people spending their life confined to bed. Stroke results in dysfunctions of motor skills, memory, and sensory perception that are caused by various kinds of ischemia leading to neuronal death. Necrotic death occurs first in the ischemic core. Neuronal necrosis in the ischemic core is caused by deprivation of oxygen, glucose, and some neurotrophic factors, and results in the release of cytotoxic substances including high-mobility group box 1.[1,2] These cytotoxic substances cause further damage to the surrounding neurons, through an activation of nonneuronal cells, astrocytes, and microglia that release other types of cytotoxic molecules, such as cytokines and nitric oxide.[3] Meanwhile in the penumbra surrounding the core, apoptosis takes over the necrosis. Considering that apoptosis has the nature of being a converging type of cell death, it is interesting to hypothesize that apoptosis plays a limited role in terminating the neuronal death expansion by necrosis.[1] In other words, the cell death mode switch from necrosis to apoptosis is a type of "Robustness" in the ischemic brain. Our major concern, however, was to identify the key molecule to exert an inhibition of necrosis, a mechanism that accomplishes the robustness or cell death mode switch. Here, we introduce the identification of prothymosin alpha (ProTα) and propose its possible robustness roles in the ischemic brain and retina through a demonstration of cellular and *in vivo* actions.

Identification of ProTα as a necrosis-inhibitory factor

The search for necrosis-inhibitory factors was initiated by the simple observation of density-dependent survival of cortical neurons in serum-free culture. After careful characterization of this unique cell death, we found that the death mode of cortical

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neurons in serum-free culture was necrosis under low-density conditions, but apoptosis under high-density conditions.[4] Therefore, we decided to search for survival factors that inhibit necrosis in the low-density culture of neurons, from the conditioned medium (CM) of high-density culture of neurons. After various approaches, the use of simple and efficient chromatographies enabled to purify active materials to a homogeneity in SDS-PAGE separation. This protein was analyzed by MALDI–TOF–MS, and a subsequent search of the nonredundant NCBI protein database for matching peptide mass fingerprints revealed 17 peptides that were unique to rat ProTα. Moreover, tandem MS analysis confirmed that the N-terminal of purified ProTα was an acetylated serine.[5] The structure of ProTα has several unique characteristics in that it is highly hydrophilic and acidic (pI = 3.55) owing to its abundance of glutamic and aspartic acids (50% of the total residues) in the middle part of the protein. The cluster of acidic amino acids in this region seems to resemble a putative histone-binding domain. A small stretch of basic residues, corresponding to thymosin-α_1,[6] is found at the N-terminal, while another stretch of basic residues at the C-terminal includes a nuclear localization signal (NLS; TKKQKK). The fact that ProTα is a monomeric protein without any regular secondary structures under physiological conditions[7] may explain its poor immunogenicity, a favorable property in terms of its clinical use. This purified protein was also biologically identified to be ProTα because a large proportion of ProTα and the survival activity in the CM were recovered in the acid-treated eluates from anti-ProTα IgG-conjugated beads, and ProTα purified to homogeneity exhibited an equivalent concentration-dependency to that of the recombinant protein. The fact that ProTα mutants lacking the N-terminal region (Δ1–29) including thymosin-α_1 or C-terminal region (Δ102–112) including the NLS retained the original activity of ProTα also indicates that ProTα itself exerts survival activity through an action on cell surface receptor.

Nonvesicular neuronal release of ProTα upon ischemic stress

ProTα was detected in CM as early as 1 h after the onset of serum-free and high-density culture. As neurons in high-density culture retain intact plasma membranes at 1 h after the start of serum-free culture and ProTα lacks a signal peptide sequence required for vesicular release, it is evident that the ProTα release occurs in a regulated and unique nonclassical manner.[8,9] However, no ProTα release was observed in the presence of serum. Taken together, these findings suggest that ProTα may play an important neuroprotective role in the event of starvation or ischemic stress.

Inhibition of necrosis by ProTα

Recombinant ProTα reversed the rapid decrease in survival of cortical neurons observed in serum-free and permanent ischemia models.[5] Addition of ProTα abolished the typical necrosis features, such as disrupted plasma membranes and swollen mitochondria in transmission electron microscope analysis at 6 h, but caused apoptosis at 12 h instead. When the cell death mode was evaluated by double staining with PI (necrosis)/annexin V (apoptosis at 3 h), PI/anti-activated-caspase-3 IgG (apoptosis at 12 h), and PI/TUNEL (apoptosis at 24 h), most of the neurons were found to die by necrosis under serum-free stress. Addition of ProTα totally switched the cell death mode from necrosis to apoptosis. Although little is known about the mechanisms of necrosis, it is only accepted that necrosis is caused by energy failure due to loss of cellular ATP.[10–13] The neuronal ATP levels and [^{3}H]-2-DG uptake rapidly decrease immediately after the start of serum-free culture, and this decrease is markedly inhibited by the addition of ProTα.[5] Quite similar changes were reproduced in the ischemic low-oxygen, low-glucose (LOG) culture. ProTα also reversed the LOG stress-induced decrease in glucose transporter (GLUT) 1/4 membrane translocation that underlies a mechanism for necrosis, or rapid decrease in ATP and [^{3}H]-2-DG uptake levels. The ProTα-induced GLUT1/4 translocation was blocked by the treatments with pertussis toxin (PTX), PLC inhibitor, PKCß inhibitor, and PKCß$_{II}$ antisense oligodeoxynucleotide (AS-ODN). Furthermore, ProTα reversed the membrane translocation of GLUT4–EGFP,[14] which had been internalized by serum deprivation in N18-RE105 hybrid cells (Fig. 1). Thus, the rescue of membrane translocation of GLUTs by ProTα through activation of putative $G_{i/o}$-coupled receptor, PLC, and PKCß$_{II}$ would be a key mechanism

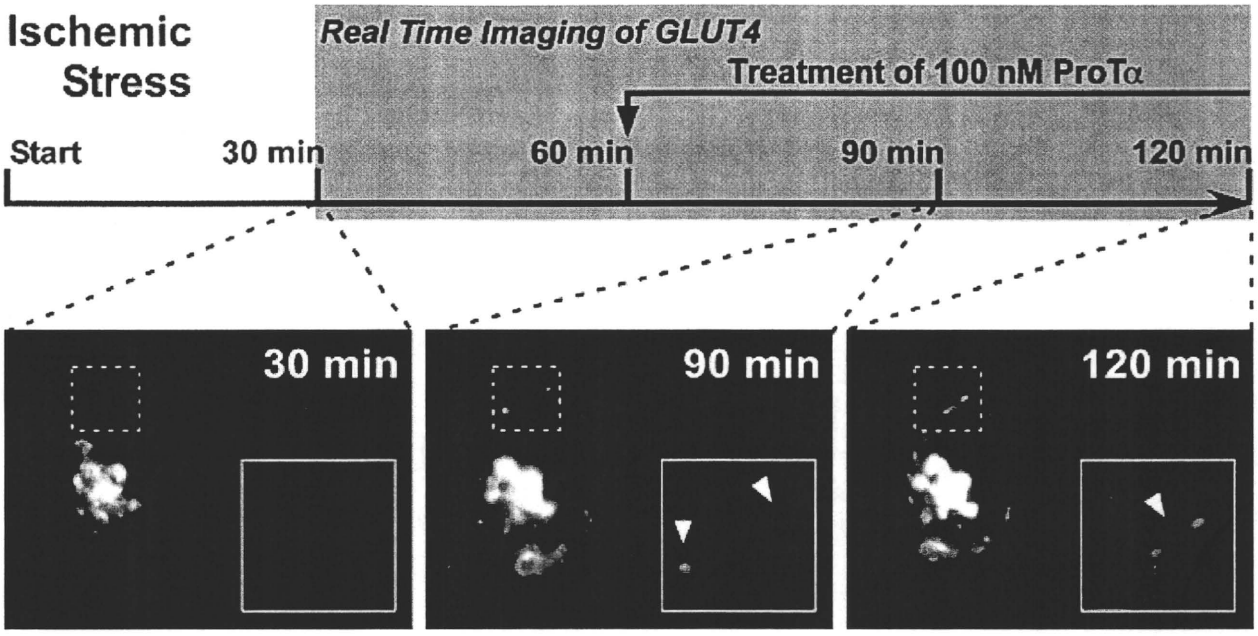

Figure 1. ProTα-induced GLUT4 translocation in N18-RE105 hybrid cells. Real-time imaging of ProTα-induced GLUT4 externalization by the use of GFP technique. The GFP images were acquired every 30 sec for 90 min (30 min after ischemic stress). GLUT4-EGFP transiently expressed in N18-RE105 cells was completely internalized 30 min after the serum deprivation, a representative ischemic stress (*left panel*). Externalization of GLUT4 was started 30 min after the treatment of ProTα (100 nM) under serum-deprivation stress (*middle panel*). ProTα recovered the membrane arrival of GLUT4 60 min after the treatment (*right panel*). Inset (*lined frame*): Higher magnifications of images for *dotted frame*. *Arrowheads* denote the membrane translocation of GLUT4.

underlying ischemia-induced necrosis or energy crisis (Fig. 2).

ProTα-induced cell death mode switch in cultured neurons

The molecular machineries for apoptosis are relatively better characterized than those for necrosis. In terms of the activation of various caspases, caspase-3 is believed to be the final execution molecule for apoptotic cell death linked to DNA breakdown and nuclear fragmentation. ProTα activates caspase-3 in serum-free and permanent ischemia models, as well as caspase-9, but not caspase-8 or caspase-12.[5] Therefore the apoptosis mechanism is mediated through mitochondrial pathway, but not through cell death receptor (caspase-8) or ER stress (caspase-12) pathway. This view was confirmed by the findings that ProTα increased the expression of pro-apoptotic Bax and Bim, while it decreased the expression of anti-apoptotic Bcl-2 and Bcl-xL, which regulate mitochondrial apoptotic signaling. On the other hand, a PKCß$_{II}$ AS-ODN reversed the ProTα-induced pro-apoptotic Bax expression. However, it should be noted that the Bax expression was also abolished by treatment with an AS-ODN for PKCß$_{I}$, but not an AS-ODN for PKCα. This fact means that the PKC involved in ProTα-induced GLUT1/4 membrane translocation underlying necrosis is more specific for ß$_{II}$-isoform. As seen in the case with necrosis, ProTα-induced Bax expression was abolished by PTX. Furthermore, we observed that ProTα causes membrane ruffling of microglia through putative $G_{i/o}$-coupled receptor, PLC, PKCß$_{I}$, and ß$_{II}$ (Ueda *et al.*, unpublished data). The ProTα-induced upregulation of Bax through PKCß$_{I}$ and ß$_{II}$ activation, seems to be consistent with the report that PKCß activation leads to Bax upregulation through NF-κB.[15,16] All these findings suggest that various cell biological actions of ProTα are mediated by the activation of $G_{i/o}$-coupled receptor, PLC, and PKC (Fig. 2). The second important issue is that ProTα switches the cell death mode by causing apoptosis.[5] As serum-free stress itself does not cause mitochondrial cytochrome c release, this stress is unlikely to drive the apoptosis

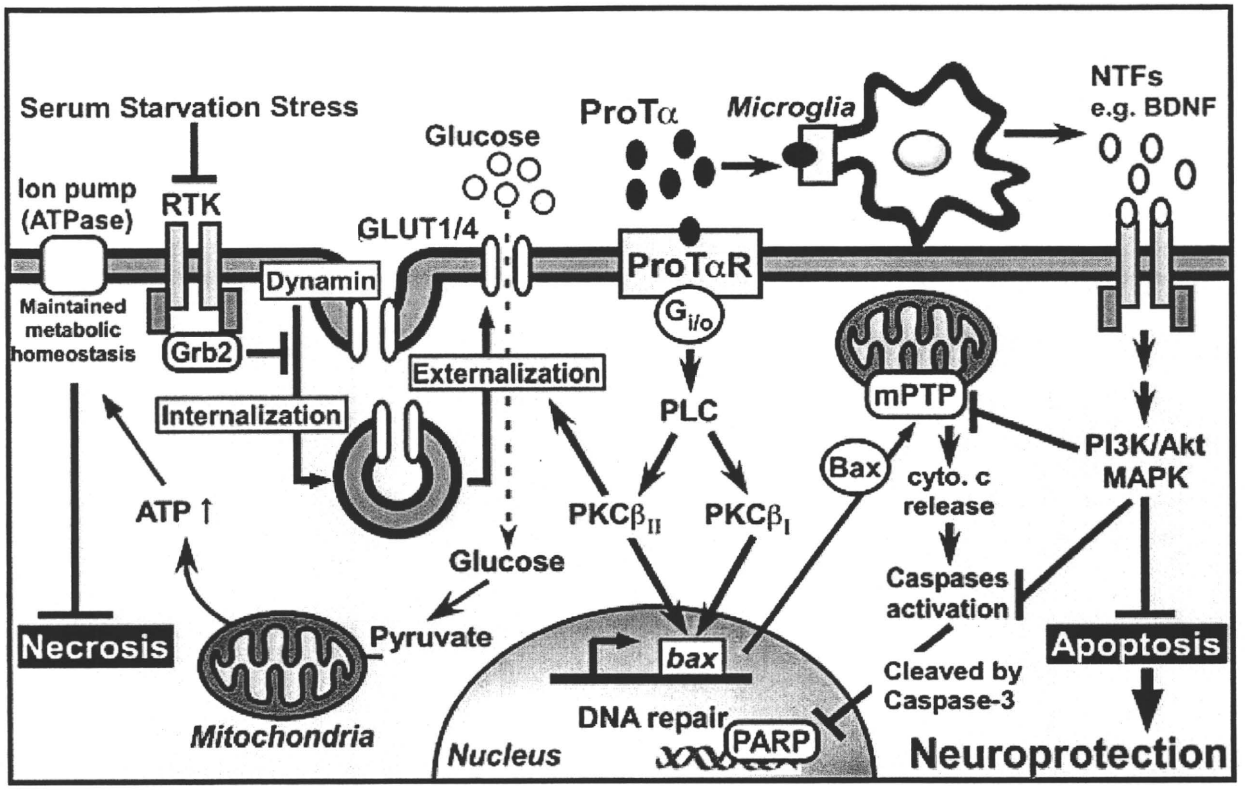

Figure 2. Cellular robustness actions of ProTα through a machinery of cell death mode switch in ischemic neurons in culture. Serum-free or starvation stress leads to endocytosis of the glucose transporters GLUT1/4, which in turn causes bioenergetic catastrophe-mediated necrosis through a rapid loss of glucose supply. Addition of ProTα to ischemia-treated neurons causes translocation of GLUT1/4 to the membrane to allow sufficient glucose supply through activation of $G_{i/o}$, PLC, and $PKC\beta_{II}$. ProTα-induced apoptosis occurs later at 12 h after the start of serum-free stress. The machinery is mediated by upregulation of Bax, which in turn causes mitochondrial cytochrome c release and subsequent apoptosis. Bax upregulation is also mediated by activation of $G_{i/o}$, PLC, and PKC, similar to the case for necrosis. However, both $PKC\beta_{I}$ and $PKC\beta_{II}$ upregulations mediate this apoptotic mechanism. As caspase-3-mediated PARP degradation minimizes the ATP consumption, the apoptosis induction may have a crucial role in inhibiting the rapid necrosis. In addition, as pyruvate, a substrate for ATP production in mitochondria, inhibits necrosis but does not cause apoptosis, the apoptosis machinery seems to be independent of the necrosis inhibition. Neurotrophins, such as BDNF or EPO, which are expected in the ischemic brain and retina, can inhibit the apoptosis machinery at a later stage.

machinery as well as the necrosis machinery. Furthermore, ProTα-induced apoptosis may not be secondary to the inhibition of necrosis, since the addition of pyruvate, which increases ATP levels through the TCA cycle, inhibited the necrosis in this culture system, but did not induce apoptosis.

Blockade of ProTα-induced apoptosis by neurotrophins

Although addition of ProTα delayed the cell death of cortical neurons in serum-free culture, most of the neurons completely died by apoptosis within 24 h. However, when neurons were treated with ProTα under conditions of ischemia and subsequent reperfusion with serum-containing medium, no significant cell death was observed for at least 48 h.[5] These findings indicate that serum factors prevented the ProTα-induced apoptosis. Indeed, further addition of nerve growth factor, brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor, or interleukin (IL)-6, comprising representative apoptosis inhibitors rescued the cell survival in serum-free culture for 48 h, while these factors alone had no effects on the survival (Fig. 2).

Inhibition of necrosis by apoptosis induction

It should be noted that concomitant addition of *N*-benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (zVAD-fmk), a pan-type caspase inhibitor, with ProTα did not lead to long-lasting survival, but caused marked cell death by necrosis at the later stage.[5] As zVAD-fmk does not affect cytochrome c release, mitochondrial bioenergetic dysfunction may lead to loss of ATP and necrosis induction. Alternatively, the blockade of caspase activity may allow a large poly(ADP-ribose)polymerase (PARP)-mediated consumption of ATP, as stated earlier (Fig. 2). Indeed we observed that the addition of PARP inhibitor 3-aminobenzamide reversed the rapid decrease in the intracellular ATP levels and inhibited necrosis after the start of the low-density and serum-free culture, while z VAD-fmk significantly reversed the increased ATP levels and necrosis inhibition by ProTα (unpublished data). In other words, apoptosis-induction in the early stage after ischemia may play a defensive role in inhibiting rapid cell death by necrosis. However, as the late phase of apoptosis is also induced by many other cytotoxic cytokines and nitric oxide through different pathways, we have to consider that the beneficial role of apoptosis in terms of antinecrosis could be limited in the case with ProTα-induced one.

Cellular roles of ProTα in cell death regulation

With regard to cell death regulation, intracellular ProTα was reported to play a cytoprotective role by inhibiting apoptosome formation in HeLa cells subjected to apoptotic stress.[17] This finding is inconsistent with reports that ProTα is released upon necrotic stress and protects against neuronal death.[5] ProTα is a highly acidic nuclear protein of the α-thymosin family, and is found in the nuclei of virtually all mammalian cells.[18,19] ProTα is generally thought to be an oncoprotein that is correlated with cell proliferation by sequestering anti-coactivator factor, a repressor of estrogen receptor activity, in various cells.[20,21] On the other hand, ProTα has been reported to act as an extracellular signaling molecule, as observed in the activation of macrophages, natural killer cells, and lymphokine-activated killer cells, and in the production of IL-2 and tumor necrosis factor-alpha (TNFα).[6] Therefore, ProTα has multiple functions inside and outside of the cell, particularly in terms of cell survival and proliferation.[22] Furthermore, the extracellular actions of ProTα seem to be cell type-specific even among brain cells, since ProTα binding is observed in neurons and microglia, but not in astrocytes.[5] The identification of ProTα-binding proteins would be the next subject.

ProTα-induced inhibition of ischemia-induced necrosis and apoptosis

Systemic administration of recombinant rat ProTα at 30 min and 3 h after focal cerebral ischemia using middle cerebral artery occlusion (MCAO) and reperfusion largely reversed the brain damage and suppressed ischemia-induced motor dysfunction and lethality.[23] Since Myc-tagged recombinant ProTα administered intraperitoneally was detected in the cortex at 3 h after MCAO stress, the neuroprotective actions of ProTα administered through systemic routes are likely to be due to transient disruption of the blood-brain barrier in the ischemic brain.[24] More details were elsewhere reported.[1] The administration of ProTα inhibited both necrotic and apoptotic cell death. When anti-BDNF or anti-erythropoietin (EPO) IgG was given into the subarachnoid space through a parietal bone, there was a reversal of ProTα-inhibited apoptosis, but not necrosis.[23] Therefore, ProTα is a unique cell death regulatory molecule, in that it converts irretrievable necrosis into controllable apoptosis. As this apoptosis can be inhibited by growth factors secreted upon ischemic stress, it is expected that ProTα may have an overall neuroprotective or robustness role in the treatment of stroke. Quite similar mechanisms were also observed in the transient global retinal ischemia model resulting from acute vascular occlusion.[25]

In vivo neuroprotective role of ProTα in a retinal ischemia model

To examine the *in vivo* role of ProTα, intravitreous pretreatment with an AS-ODN against ProTα was carried out. This AS-ODN, but not a mismatched scrambled oligodeoxynucleotide (MS-ODN), significantly worsened the histological damage at day 4 after retinal ischemic stress. Similar results were observed for intravitreous pretreatment (1 μg/eye, 30 min prior to ischemic stress) of anti-ProTα IgG,

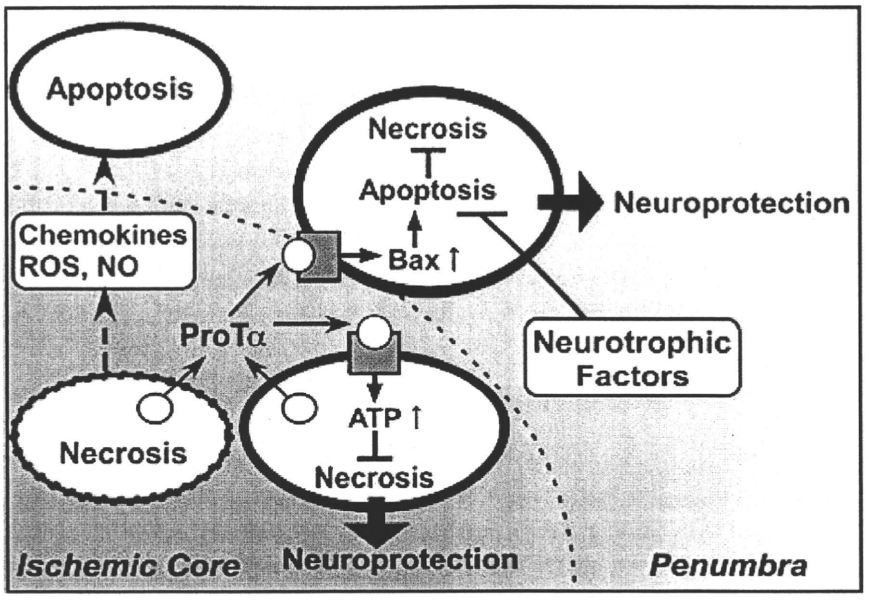

Figure 3. Robustness roles of ProTα in the neuroprotection in stroke. The hypothesis depicts the machineries underlying the robustness roles of ProTα through a mechanism of cell death mode switch in cerebral ischemia. In the mild cerebral ischemia, ProTα is first released upon ischemic stress from necrotic cells in the ischemic core. Released ProTα exerts a suppression of the necrosis of neighbored neurons, which plays a role of the early stage of robustness. ProTα at the same time causes apoptosis machineries including caspase 3 activation, which in turn delays the necrosis (through a PARP degradation), as another type of robustness. Expression of neurotrophic factors, such as BDNF or EPO will then occur and block the apoptosis in the penumbra (the late stage of robustness). As the robustness actions of endogenous ProTα seem to be insufficient in the intense ischemia, exogenous administration of ProTα is required for the cure of ischemic brain damages.

which absorbs ProTα.[25] Functional damage was also deteriorated by this antibody treatment, as evaluated by electroretinography (ERG). As ProTα-like immunoreactivities completely disappeared without exception at 3 h after the stress, it is evident that ProTα released upon ischemic stress plays *in vivo* neuroprotective roles.

Conclusions

The discovery of ProTα initiated investigations into what happens in the event of neuronal necrosis, followed by searches for compounds that inhibit necrosis, based on the detection of its presence in CM from neuronal cultures. The hypothesis that ProTα acts as a "Robustness" or cell death mode switch molecule from uncontrollable necrosis to neurotrophin-reversible apoptosis may provide a promising new strategy for preventing serious damage in stroke (Fig. 3). However, the complete clarification of mechanisms underlying potent neuroprotective actions of ProTα in cerebral and retinal ischemia should wait for the identification of the specific receptor. From this point of view, the involvement of unknown mechanisms independent of $G_{i/o}$-coupled receptors may not be excluded at present. Regarding clinical issues, it is evident that recombinant ProTα itself has unique and potent therapeutic potentials against acute stroke.

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Conflicts of interest

The authors declare no conflicts of interest.

References

1. Ueda, H. 2009. Prothymosin alpha and cell death mode switch, a novel target for the prevention of cerebral ischemia-induced damage. *Pharmacol. Ther.* **123:** 323–333.
2. Qiu, J. *et al.* 2008. Early release of HMGB-1 from neurons after the onset of brain ischemia. *J. Cereb. Blood Flow Metab.* **28:** 927–938.
3. Lai, A.Y. & K.G. Todd. 2006. Microglia in cerebral ischemia: molecular actions and interactions. *Can. J. Physiol. Pharmacol.* **84:** 49–59.
4. Fujita, R. *et al.* 2001. Cell density-dependent death mode switch of cultured cortical neurons under serum-free starvation stress. *Cell. Mol. Neurobiol.* **21:** 317–324.
5. Ueda, H. *et al.* 2007. Identification of prothymosin-alpha1, the necrosis-apoptosis switch molecule in cortical neuronal cultures. *J. Cell Biol.* **176:** 853–862.
6. Pineiro, A., O.J. Cordero & M. Nogueira. 2000. Fifteen years of prothymosin alpha: contradictory past and new horizons. *Peptides* **21:** 1433–1446.
7. Gast, K. *et al.* 1995. Prothymosin alpha: a biologically active protein with random coil conformation. *Biochemistry* **34:** 13211–13218.
8. Matsunaga, H. & H. Ueda. 2006. Voltage-dependent N-Type Ca2+ channel activity regulates the interaction between FGF-1 and S100A13 for stress-induced non-vesicular release. *Cell Mol. Neurobiol.* **26:** 237–246.
9. Matsunaga, H. & H. Ueda. 2006. Evidence for serum-deprivation-induced co-release of FGF-1 and S100A13 from astrocytes. *Neurochem. Int.* **49:** 294–303.
10. Eguchi, Y., S. Shimizu & Y. Tsujimoto. 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res.* **57:** 1835–1840.
11. Fujita, R. & H. Ueda. 2003. Protein kinase C-mediated cell death mode switch induced by high glucose. *Cell Death Differ.* **10:** 1336–1347.
12. Fujita, R. & H. Ueda. 2003. Protein kinase C-mediated necrosis-apoptosis switch of cortical neurons by conditioned medium factors secreted under the serum-free stress. *Cell Death Differ.* **10:** 782–790.
13. Zong, W.X. & C.B. Thompson. 2006. Necrotic death as a cell fate. *Genes Dev.* **20:** 1–15.
14. Hasegawa, T. *et al.* 2007. Differential localization of aquaporin-2 and glucose transporter 4 in polarized MDCK cells. *Histochem. Cell Biol.* **127:** 233–241.
15. Herrmann, O. *et al.* 2005. IKK mediates ischemia-induced neuronal death. *Nat. Med.* **11:** 1322–1329.
16. Mattson, M.P. & S. Camandola. 2001. NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J. Clin. Invest.* **107:** 247–254.
17. Jiang, X. *et al.* 2003. Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. *Science* **299:** 223–226.
18. Clinton, M. *et al.* 1991. Evidence for nuclear targeting of prothymosin and parathymosin synthesized *in situ*. *Proc. Natl. Acad. Sci. USA* **88:** 6608–6612.
19. Haritos, A.A. *et al.* 1985. Primary structure of rat thymus prothymosin alpha. *Proc. Natl. Acad. Sci. USA* **82:** 343–346.
20. Bianco, N.R. & M.M. Montano. 2002. Regulation of prothymosin alpha by estrogen receptor alpha: molecular mechanisms and relevance in estrogen-mediated breast cell growth. *Oncogene* **21:** 5233–5244.
21. Martini, P.G. *et al.* 2000. Prothymosin alpha selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. *Mol. Cell Biol.* **20:** 6224–6232.
22. Gomez-Marquez, J. 2007. Function of prothymosin alpha in chromatin decondensation and expression of thymosin beta-4 linked to angiogenesis and synaptic plasticity. *Ann. New York Acad. Sci.* **1112:** 201–209.
23. Fujita, R. & H. Ueda. 2007. Prothymosin-alpha1 prevents necrosis and apoptosis following stroke. *Cell Death Differ.* **14:** 1839–1842.
24. Paul, R. *et al.* 2001. Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. *Nat. Med.* **7:** 222–227.
25. Fujita, R. *et al.* 2009. Prothymosin-alpha plays a defensive role in retinal ischemia through necrosis and apoptosis inhibition. *Cell Death Differ.* **16:** 349–358.

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Stress-induced non-vesicular release of prothymosin-α initiated by an interaction with S100A13, and its blockade by caspase-3 cleavage

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The nuclear protein prothymosin-α (ProTα), which lacks a signal peptide sequence, is released from neurons and astrocytes on ischemic stress and exerts a unique form of neuroprotection through an anti-necrotic mechanism. Ischemic stress-induced ProTα release is initiated by a nuclear release, followed by extracellular release in a non-vesicular manner, in C6 glioma cells. These processes are caused by ATP loss and elevated Ca^{2+}, respectively. S100A13, a Ca^{2+}-binding protein, was identified to be a major protein co-released with ProTα in an immunoprecipitation assay. The Ca^{2+}-dependent interaction between ProTα and S100A13 was found to require the C-terminal peptide sequences of both proteins. In C6 glioma cells expressing a Δ88–98 mutant of S100A13, serum deprivation caused the release of S100A13 mutant, but not of ProTα. When cells were administered apoptogenic compounds, ProTα was cleaved by caspase-3 to generate a C-terminal peptide-deficient fragment, which lacks the nuclear localization signal (NLS). However, there was no extracellular release of ProTα. All these results suggest that necrosis-inducing stress induces an extacellular release of ProTα in a non-vesicular manner, whereas apoptosis-inducing stress does not, owing to the loss of its interaction with S100A13, a cargo molecule for extracellular release.
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Prothymosin-α (ProTα), a nuclear protein, is widely distributed throughout the body and has various intracellular functions in determining life and death.[1,2] ProTα bearing a conventional nuclear localization signal (NLS) is largely localized in the nucleus, and has important functions in the regulation of cell differentiation and proliferation.[1–3] When a cell is subjected to apoptotic stimuli, on the other hand, ProTα is released from the nucleus to the cytosol in which it inhibits apoptosome formation.[4] Thus, ProTα is supposed to have a proliferative function in the nucleus under normal conditions, and a self-defensive function in the cytosol under apoptotic conditions.

We have recently identified ProTα as a unique anti-neuronal necrosis factor in the conditioned medium (CM) of cortical neurons,[5] and discovered its potent neuroprotective functions in cerebral and retinal ischemia.[6,7] This protein is extracellularly released on starving or ischemic stress, and inhibits necrosis by inducing the membrane translocation of glucose transporters, which are endocytosed under ischemic conditions, resulting in an acceleration of necrosis owing to energy crisis.[5] On the basis of pharmacological analyses, ProTα-induced translocation of glucose transporters is mediated by stimulation of a putative $G_{i/o}$-coupled receptor, phospholipase C, and protein kinase C (PKC) β_{II}. This fact indicates that ProTα has another self-defensive function as an extracellular signal under conditions inducing necrosis.

However, little is known of the mechanisms underlying extracellular ProTα release. Of importance are the facts that the majority of ProTα content in the nucleus is extracellularly released on stress, before membrane disruption,[5] and that ProTα lacks a signal peptide sequence, which is required for sorting to the endoplasmic reticulum (ER)-Golgi system before exocytosis. Therefore, ProTα release seems to proceed in a non-classical or non-vesicular manner under ischemic stress conditions. Here, we report the ischemia-induced nuclear release of ProTα, followed by its interaction with S100A13, a cargo molecule for extracellular release. We also discuss the mechanism underlying the lack of extracellular ProTα release under apoptotic conditions.

Results

Serum-deprivation stress-induced non-classical release of ProTα. ProTα is exclusively localized in the nuclei of various cells, including neurons.[1–3] Starvation stress caused by serum deprivation induced a disappearance of ProTα from neurons and astrocytes, as early as 3 h after the start of primary culture (Figure 1a). Recently, we discovered the release of this protein into the CM of cultured cortical neurons under serum-free starving conditions causing necrosis.[5]

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Keywords: non-vesicular release; prothymosin-α; S100A13; necrosis and apoptosis
Abbreviations: BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CM, conditioned medium; DAMPs, damage-associated molecular patterns; 2-DG, 2-deoxy-D-glucose; ER, endoplasmic reticulum; FGF, fibroblast growth factor; FRET, fluorescence resonance energy transfer; HMGB1, high-mobility group box1; NLS, nuclear localization signal; OU, oscillation unit; PKC, protein kinase C; ProTα, prothymosin-α; QCM, quartz crystal microbalance
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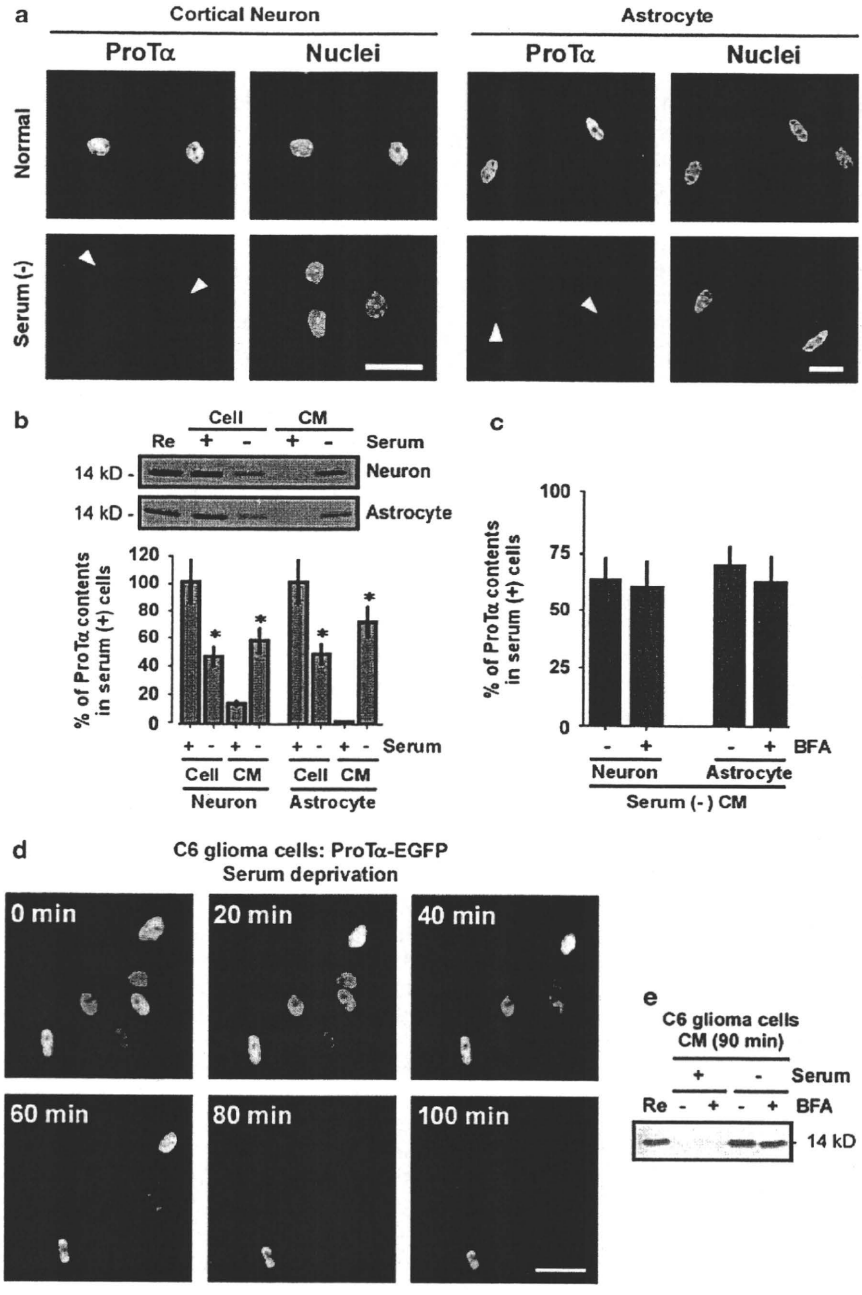


Figure 1 Serum-deprivation stress induced the brefeldin-A-insensitive non-classical extracellular release of ProTα. (**a**) Altered distribution of ProTα in cortical neurons and astrocytes. Cells were fixed for immunocytochemistry 3 h after serum-deprivation stress, indicated as serum (−). Arrowheads denote the nuclei of cells showing ProTα release. (**b**) ProTα release in cortical neurons and astrocytes induced by serum (−) stress. ProTα, a highly acidic protein, was purified from 5×10^5 cells and their CM using a phenol extraction procedure, and visualized with Coomassie brilliant blue. Data represent the means ± S.E.M. of five independent experiments [$^*P = 0.01$, *versus* the corresponding serum (+) treatment]. (**c**) Characterization of brefeldin A-insensitive non-classical extracellular release of ProTα. Brefeldin A (BFA; 8 μg/ml) was added to the culture of cortical neurons and astrocytes 12 h before serum deprivation. CM samples ($n = 5$) were used for the purification of ProTα. (**d**) Real-time imaging of serum-deprivation stress-induced ProTα release. ProTα-EGFP stably expressed in C6 glioma cells was released on serum-deprivation stress. (**e**) Serum-deprivation stress-induced ProTα release in a BFA-insensitive manner. CM samples from C6 glioma cells culture were collected at 90 min after serum-deprivation stress (90 min). Scale bars represent 20 μm. Re: recombinant rat ProTα

As shown in Figure 1b, when serum-derivation stress was given to neurons or astrocytes, the amounts of ProTα in both cell types markedly decreased at 3 h. On the other hand, the ProTα levels in the CM of both cell types in the presence of serum were negligible. However, the serum-deprivation stress caused a significant extracellular release of ProTα from both cell types. Brefeldin A, a blocker of protein transport from the ER to the Golgi apparatus, did not affect

ProTα release (Figure 1c), suggesting that the manner of release differs from conventional vesicular release. Extracellular release of ProTα was also observed in a rat astroglial C6 glioma cell line after serum deprivation. In C6 glioma cells expressing ProTα-EGFP, the serum-deprivation stress-induced decrease of fluorescence in nuclei started as early as 20 min after serum deprivation, and complete disappearance was observed at 80 min, although a small population of cells (below 5%) still retained fluorescence (Figure 1d). However, no significant fluorescence signal was observed in the cytosol at these time points, suggesting that stress-induced nuclear export of ProTα is a rate-limiting step in the non-classical extracellular release of ProTα. As seen with primary neurons and astrocytes, brefeldin A did not affect the serum-deprivation stress-induced release of native ProTα release from C6 glioma cells (Figure 1e).

ATP-dependent nuclear localization of ProTα. We have earlier reported that serum-free starvation stress causes a rapid decrease in cellular ATP levels, leading to necrosis and extracellular ProTα release from the nucleus.[5] As shown in Figure 2a, the addition of 2-deoxy-D-glucose (2-DG) to cultured C6 glioma cells in serum-containing medium caused a rapid decrease in ATP levels. When the subcellular localization of ProTα was examined (Figure 2b), the addition of 2-DG to cells without serum-starvation stress decreased the number of cells showing nuclear localization of ProTα, and increased the number showing cytosol localization. However, no significant extracellular ProTα release was observed. On the other hand, serum-deprivation treatment decreased the number of cells showing nuclear localization and increased the number showing extracellular release. Thus, these results suggest that the loss of cellular ATP induces the transport of ProTα from the nucleus to cytosol, but is not sufficient to cause extracellular release of ProTα from the cytosol.

We studied the machinery underlying nucleus-to-cytosol export. When Alexa Fluor488-labeled ProTα and Alexa Fluor568-bovine serum albumin (BSA) were co-injected into the cytosols of C6 glioma cells, Alexa Fluor488-ProTα was rapidly localized to the nucleus, within 10 min, whereas Alexa Fluor568-BSA remained in the cytosol (Figure 2c, upper left 4 panels). Treatment of cells with 2-DG abolished the nuclear localization of Alexa Fluor488-ProTα and redistributed it throughout the cell (Figure 2c, upper middle 4 panels). This re-distribution was completely reversed by co-injection of ATP (Figure 2c, upper right 4 panels). On the other hand, serum deprivation caused a re-distribution of ProTα from nucleus to cytosol, but did not result in extracellular release in the presence of amlexanox, which inhibits the release of proteins lacking a signal peptide sequence[8–11] (Figure 2c, lower panels). Similarly, co-injection of ATP reversed the nucleus-to-cytosol export. Thus, it is evident that nuclear localization of ProTα is closely related to the cellular ATP level.

Importin α has an important function in the nuclear localization of proteins possessing an NLS. For the sustained localization of such proteins, importin α should be released into the cytosol for repeated use. The GTP-bound form of Ran, a small GTP-binding protein, is known to execute this importin recycling process.[12] Therefore, the loss of cellular ATP is expected to impair the nuclear localization of ProTα owing to difficulty maintaining the GTP-bound state of Ran. Indeed, the nuclear localization of ProTα-EGFP was clearly impaired by injection of anti-importin α IgG into C6 glioma cells (Figure 2d). However, the nuclear levels of ProTα were not affected by leptomycin B, an inhibitor of the nuclear export receptor CRM1 (data not shown). When wheat germ agglutinin, an inhibitor of the nuclear pore complex, was injected into the nuclei of C6 glioma cells, ProTα was redistributed throughout the cells (Figure 2e). All of these findings suggest that ProTα is localized in the nucleus through an ATP-dependent importin-NLS mechanism,[13] and that nucleus-to-cytosol export owing to loss of ATP occurs through passive diffusion.

Stress-induced extracellular co-release of ProTα with S100A13. When the CM from serum-deprived C6 glioma cells was immunoprecipitated with anti-ProTα IgG, two significant protein bands were stained by CBB (Figure 3a). The upper band was identified as ProTα by immunoblot using an acidic protein transfer procedure.[14] The lower band, at approximately 10 kDa, was identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (five peptides, 57.1% coverage) followed by MS/MS sequence-tag analysis using the NCBI protein database, as S100A13, a member of the Ca^{2+}-binding S100 family.[15–17] Immunoblotting also confirmed that this protein is identical to S100A13.

As shown in Figure 3b, naturally occurring ProTα is localized within nuclei, whereas S100A13 is evenly distributed throughout cells. When cultured cells were deprived of serum, both ProTα and S100A13 were completely lost from cells at the time point of 3 h. The cellular loss of S100A13 and ProTα was also blocked by amlexanox, a potent inhibitor of S100A13.[8–11,18] Quantitative immunoblot analysis confirmed that amlexanox abolished the serum-deprivation stress-induced extracellular release of ProTα (Figure 3c).

Protein–protein interaction between ProTα and S100A13. The interaction between ProTα and S100A13 was characterized by use of various deletion mutants of both proteins. In this study, GST-tagged ProTα and *Strep*-tagII-S100A13 were used (Figure 4a). The addition of *Strep*-tagII-S100A13 to GST-ProTα immobilized on the sensor tip of a quartz crystal microbalance (QCM) decreased the quartz oscillation, as quantified by the oscillation unit (OU: ΔF in Hz), which represents the degree of interaction between the two proteins, as reported earlier.[19] The interaction between proteins was enhanced in the presence of Ca^{2+}, but not in the presence of Mg^{2+} or Cu^{2+}, and this enhancement was Ca^{2+} dependent in the range 0.1–200 μM (Table 1; Figure 4b). As the Ca^{2+}-dependent interaction was further enhanced by the addition of Cu^{2+} (Table 1), which has substantial binding affinity for S100A13, we used the addition of both Ca^{2+} and Cu^{2+} (100 μM and 100 nM, respectively) to determine the best ionic conditions for the interaction between GST-ProTα and *Strep*-tagII-S100A13. From the kinetic analysis,[19] the saturated OU_{max} for the interaction of *Strep*-tagII-S100A13 with immobilized GST-ProTα (430 fmol) was 201.44 ± 3.53 OU, which corresponds to

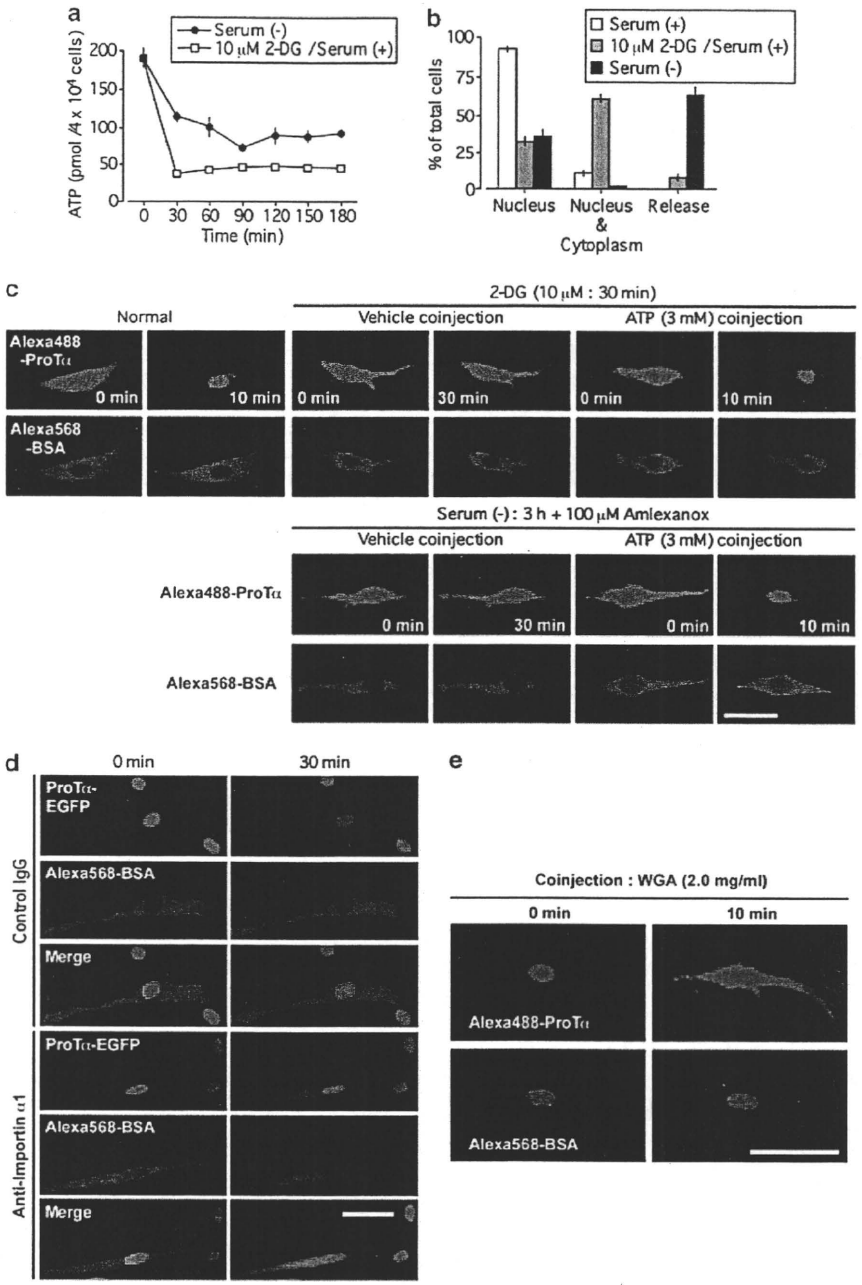


Figure 2 Mechanism underlying the nuclear transport of ProTα. (**a**) Decrease in intracellular ATP levels induced by serum-deprivation stress. Time courses of intracellular ATP levels were measured in ProTα-EGFP stably expressing C6 glioma cells subjected to serum-deprivation stress or treated with 2-DG (10 μM, with serum). (**b**) Altered distribution of ProTα-EGFP by serum (−) or 2-DG treatments (3 h). Distribution of ProTα-EGFP was measured after fixation of C6 glioma cells and visualization of nuclei with Hoechst 33342. Data represent the means ± S.E.M. of 4–6 independent experiments. (**c**) ATP-induced recovery of nuclear import of ProTα after 2-DG treatment or serum (−) stress in the presence of amlexanox (100 μM). Alexa Fluor488-ProTα and Alexa Fluor568-BSA, with or without ATP (3 mM in a needle), were co-injected into cytoplasm. (**d**) Nuclear import of ProTα mediated by importin α. Importin α, a nuclear transport receptor, binds to classical NLS-containing proteins and links them to the nuclear pore complex. Re-distribution of ProTα-EGFP after cytosolic co-injection of anti-importin α IgG (0.1 μg/ml in a needle) and Alexa Fluor568-BSA. (**e**) Passive diffusion of ProTα as a nuclear export mechanism. Injection of wheat germ agglutinin (WGA, 2.0 mg/ml in a needle) into the nucleus induced a re-distribution of ProTα throughout the cell. Scale bars represent 20 μm

435.63 ± 7.63 fmol. Therefore, it is evident that ProTα and S100A13 interact at a ratio of 1 : 1.

The C-terminal domains of several types of S100 family proteins are reported to interact with various target proteins.[16] Among these proteins, S100A13 has a unique C-terminal 11-amino-acid sequence (RKEKVLAIRKK), which contains as many as six basic amino acids. In the QCM analysis, *Strep*-tagII-S100A13 lacking this C-terminus (amino-acids

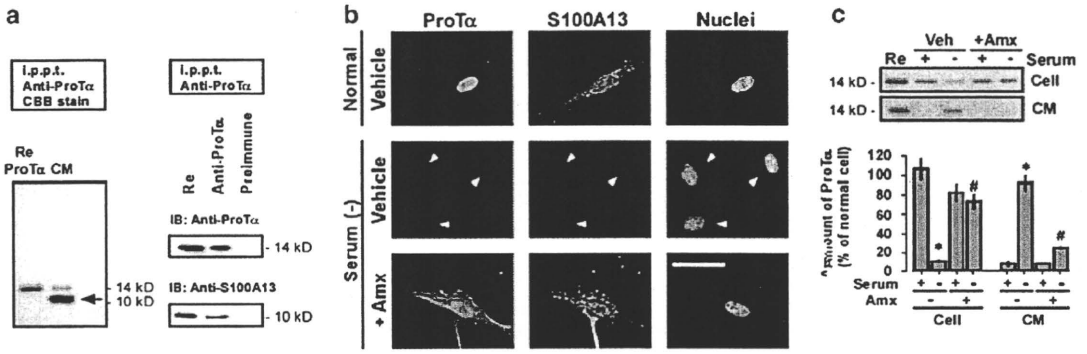


Figure 3 Identification of S100A13 co-released with ProTα on serum-deprivation stress. (**a**) S100A13 is a major protein interacting with extracellular ProTα. CM from serum-deprived C6 glioma cells (3 h) was subjected to immunoprecipitation with anti-ProTα or pre-immune IgG. Lower band was identified as S100A13 (arrow). Re, recombinant rat ProTα; IB, immunoblot. (**b**) Blockade of stress-induced extracellular release of ProTα and S100A13 by amlexanox. Cells were subjected to serum-deprivation stress (3 h) in the presence or absence of amlexanox (Amx: 100 μM, pretreatment for 30 min). Arrowheads denote the nuclei of cells showing ProTα and S100A13 release. Scale bar represents 20 μm. (**c**) Biochemical analysis of amlexanox-induced blockade of serum-deprivation stress-induced extracellular release of ProTα. ProTα in the CM and residual cells was measured by immunoblot. CM was immunoprecipitated with anti-ProTα IgG. Data represent the means ± S.E.M. of five independent experiments [* and $^{\#}P<0.01$, *versus* the corresponding serum (+) and the corresponding Amx (−) treatment, respectively]

88–98) showed no significant interaction with ProTα (Figure 4c). On the other hand, in the experiments using immobilized *Strep*-tagII-S100A13, the C-terminal deletion mutants ProTα (Δ79–112) and ProTα (Δ102–112) showed decreased potency for interaction (association rate constant: k_a), whereas the N-terminal deletion mutants ProTα (Δ1–68) and ProTα (Δ1–86) did not (Figure 4d). As the C-terminal peptide ProTα (amino-acids 102–112; TKKQKKTDEDD), but not its reverse peptide (DDEDTKKQKKT), inhibited the interaction between GST-ProTα and *Strep*-tagII-S100A13 (Figure 4e), the C-terminal sequence appears to have a crucial function in this interaction. On the contrary, the N-terminal region is presumed to have an unidentified regulatory function in this interaction.

Interaction between ProTα and S100A13 is required for the extracellular release of ProTα. To confirm that the stress-induced extracellular release of ProTα depends on the ProTα–S100A13 interaction, we established C6 glioma cells stably expressing *Strep*-tagII-S100A13 Δ88–98, which lacks affinity for ProTα. In the immunocytochemical study, serum-deprivation stress caused an extracellular release of both ProTα and *Strep*-tagII-S100A13 full-length mutant from C6 glioma cells (Figure 5a, left panels, and 5b). However, with the *Strep*-tagII-S100A13 Δ88–98 mutant, serum-deprivation stress caused the release of the mutant protein, but not ProTα (Figure 5a, right panels, and 5b). This finding suggests that the C-terminal basic residue-rich domain of S100A13 is required for the interaction with ProTα in living cells, and that S100A13 has a function as a cargo molecule involved in the extracellular export of ProTα. To identify the intracellular locales of interaction between ProTα and S100A13, we performed *Strep*-tagII pull-down assay using nuclear and cytosolic fractions from C6 glioma cells stably expressing *Strep*-tagII-S100A13 (Figure 5c). In the absence of stress, there was no interaction between *Strep*-tagII-S100A13 and ProTα in either nuclear or cytosol fraction. Under the serum-deprivation stress, however, a significant interaction was observed in the cytosol fraction, but not in the nuclear one. The level of co-precipitated ProTα in the cytosol was decreased in a time-dependent manner in the range between 1 and 3 h, possibly because of the loss of cytosolic ProTα by extracellular release of both proteins. To evaluate the ProTα–S100A13 interaction in living cells, we performed fluorescence resonance energy transfer (FRET) analysis. However, the representative FRET analysis using a pair of CFP and YFP was not successful, because the YFP-fused ProTα showed an abnormal distribution. Instead, we attempted to assess the interaction between ProTα-EGFP and DsRed2-S100A13 in the presence of amlexanox, which has no direct effect on their interaction (Table 1). In addition, we performed using phenol red-free DMEM, which does not decrease the survival activity of C6 glioma cells under serum-deprivation condition (Supplementary Figure S1). In the cell population, FRET analysis in the presence of amlexanox, the serum-deprivation stress-induced interaction between ProTα and S100A13 (Figure 5d), and ProTα and S100A13 were redistributed throughout the cell (Figure 5g). To calculate the FRET efficiency, we performed acceptor photo-bleaching. When the acceptor (DsRed2-S100A13) was earlier bleached, serum-deprivation stress did not cause elevation of FRET ratio; however, ProTα and S100A13 were also redistributed throughout the cell (Figure 5d and g). Next, we performed the single cell FRET analysis in the absence of amlexanox. As shown in Supplementary Figure S2a, the FRET ratio in the cytosol was rapidly increased at 80 min after serum deprivation, whereas then gradually decreased, possibly because of the extracellular release of ProTα–S100A13 complex. In accord with this observation, the fluorescence intensity derived from donor ProTα-EGFP decreased at 80 min, whereas the one derived from acceptor DsRed2-S100A13 transiently increased (Supplementary Figure S2b). These results strongly suggest that the interaction between ProTα-EGFP and DsRed2-S100A13 occurs in the cytosol. As shown in Figure 5e, the serum-deprivation stress-induced interaction

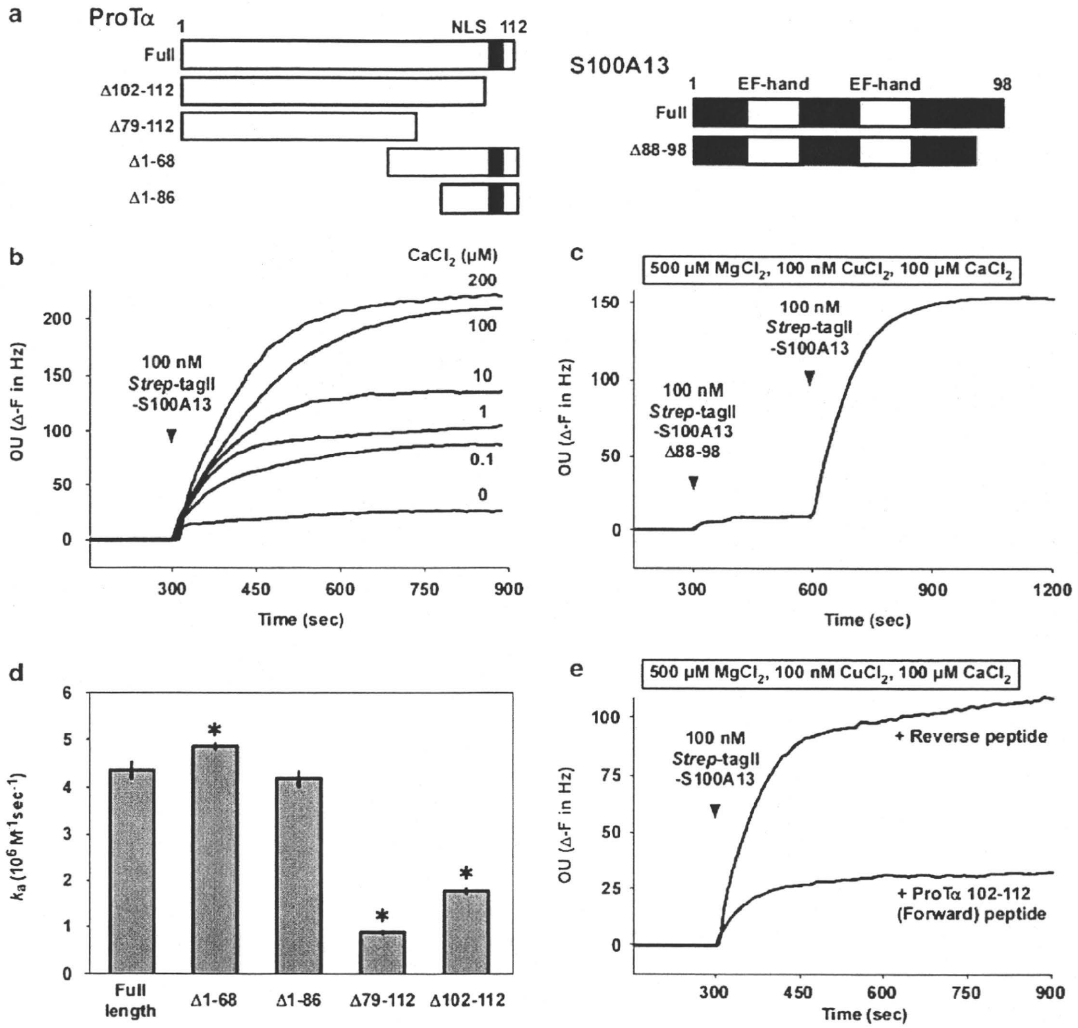


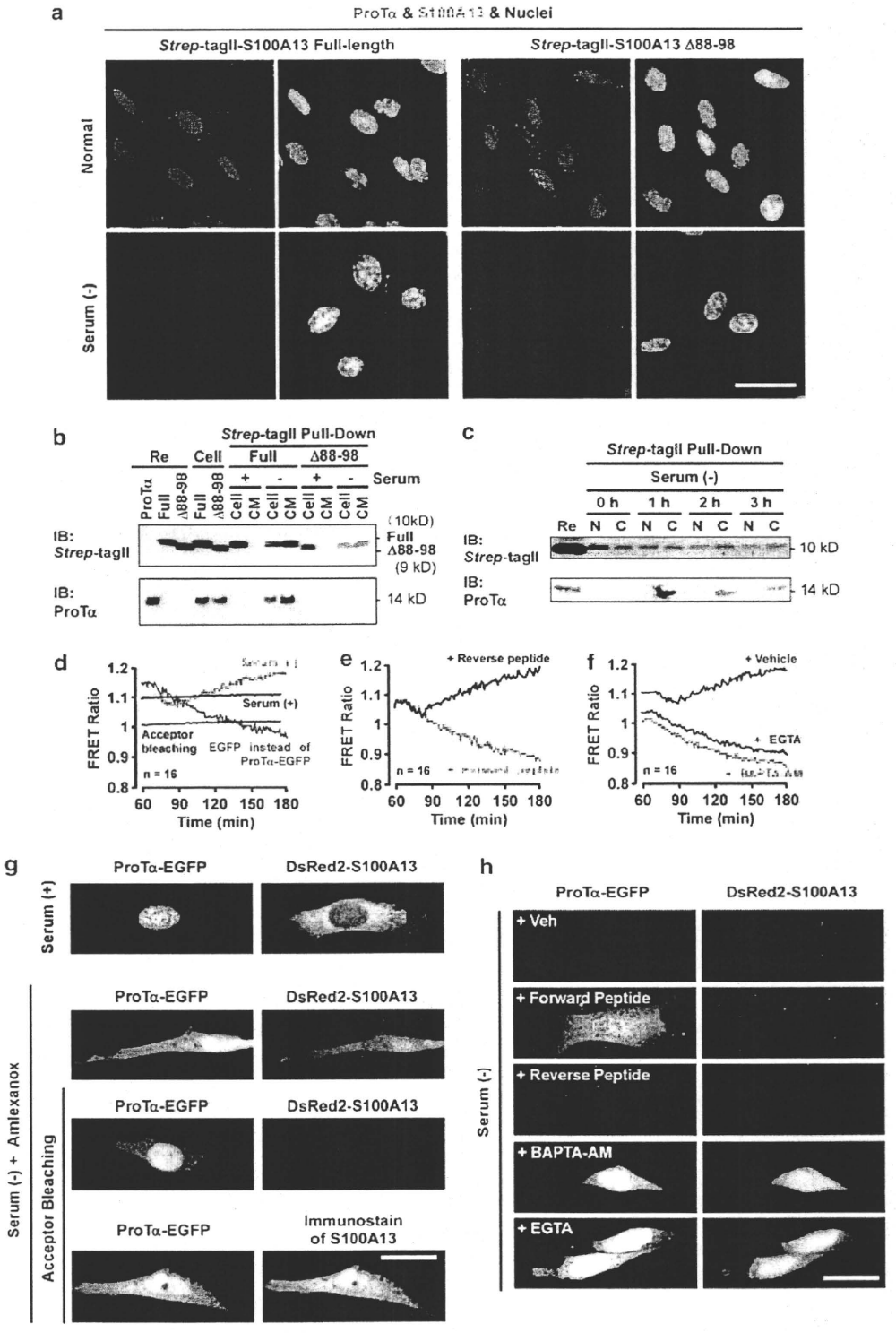
Figure 4 Biochemical interaction between GST-ProTα and *Strep*-tagII-S100A13, evaluated by QCM. (**a**) ProTα and S100A13 mutant constructs. (**b**) The interaction between GST-ProTα and *Strep*-tagII-S100A13 occurs in a Ca^{2+} concentration-dependent manner. The time course of the interaction in the presence of various concentrations of Ca^{2+} (0–200 μM) was measured. (**c**) Loss of interaction between S100A13 lacking the C-terminal region (Δ88–98) and ProTα. (**d**) Loss of interaction between GST-ProTα deletion mutants lacking C-terminal regions and *Strep*-tagII-S100A13. Data represent the means ± S.E.M. of five independent experiments (*$P<0.05$, *versus* full length). (**e**) Interference by ProTα C-terminal forward peptide (amino-acids 102–112). Arrowheads represent the application points of *Strep*-tagII-S100A13 mutants

Table 1 The association rate constant (k_a) and dissociation constant (K_D) value of *Strep*-tagII-S100A13 for GST-ProTα in the QCM assay

	Mg^{2+} (μM)	Ca^{2+} (μM)	Cu^{2+} (μM)	Amlexanox (100 μM)	k_a (10^4/M/s)	K_D (10^{-8} M)
A	0	0	0	–	1.98 ± 0.28	21.19 ± 3.43
B	100	0	0	–	1.83 ± 0.31	27.66 ± 6.52
C	0	100	0	–	10.03 ± 0.14**	6.98 ± 0.09**
D	0	0	100	–	3.40 ± 0.52	13.62 ± 3.03
E	500	100	0	–	7.01 ± 0.21	5.72 ± 0.17
F	500	100	0.1	–	9.46 ± 0.23*	3.20 ± 0.68*
G	500	100	0	+	6.84 ± 0.06	5.89 ± 0.18
H	500	100	0.1	+	9.88 ± ± 0.43*	4.38 ± 0.20*

The interaction between ProTα and S100A13 was in a Ca^{2+}-dependent manner. The interaction was analyzed by both GST-ProTα and *Strep*-tagII-S100A13. GST-ProTα was immobilized on the anti-GST antibody-coated sensor chip, hence orientation of ProTα (host sample) was fixed. The k_a and K_D values were obtained by the analysis using cumulative application of *Strep*-tagII-S100A13 (guest sample). Cu^{2+} potentiated the Ca^{2+}-dependent interaction between GST-ProTα and *Strep*-tagII-S100A13. Amlexanox did not affect this interaction. Each experiment was analyzed in interaction buffer. The data shown are mean ± S.E.M. of 3–5 independent experiments. *$P<0.05$ and **$P<0.01$ *versus* corresponding control (A and E)

between ProTα and S100A13 was abolished by the C-terminal (102–112) peptide of ProTα, but not by the reverse peptide. The treatment with C-terminal peptide inhibited the serum-deprivation-induced extracellular release of ProTα, but not S100A13, whereas the reverse peptide did not inhibit the extracellular release of either protein (Figure 5h, upper 6 panels).



Ca^{2+} influx is involved in the extracellular release of ProTα based on the interaction with S100A13. In the FRET analysis (Figure 5f), the serum-deprivation stress-induced interaction between ProTα and S100A13 was abolished when the cells were treated with EGTA or BAPTA-AM, extracellular and intracellular Ca^{2+}-chelating agents, respectively. The serum-deprivation-induced loss of these proteins was also abolished by EGTA or BAPTA-AM (Figure 5h, lower 4 panels).

Caspase-catalyzed cleavage of ProTα inhibits the stress-induced extracellular release. When C6 glioma cell culture was carried out in various concentrations of serum, significant depletion of ProTα was observed in the absence of serum, but not in the presence of 2 or 10% serum (Figure 6a, upper 6 panels, and 6b). When staurosporine, tunicamycin, and etoposide, which are known to induce apoptosis, were added to 2% serum medium, ProTα was redistributed throughout the cell (Figure 6a, middle 6 panels). However, these apoptogenic compounds did not cause any significant extracellular release of ProTα, but led to the production of large amounts of a smaller ProTα fragment (Figure 6b). These re-distribution and fragmentation of ProTα were in a caspase-3 inhibitor zDEVD-fmk-reversible manner (Figure 6a, lower 6 panels and 6b). On the other hand, these compounds did not affect intracellular ATP levels (Supplementary Figure S3).

When rat ProTα was treated with active caspase-3, a 13 kDa protein band was time dependently generated in a zDEVD-fmk-reversible manner (Figure 6c). This finding is consistent with reports that human ProTα has three over-lapping caspase-3 cleavage sites, 94DDED97, 95DEDD98, and 97DDVD101,[20,21] immediately upstream of the NLS moiety in its C-terminus, and that this fragment lacks an NLS moiety KKQK, which is present in rat ProTα at amino-acid positions 103–106. To identify the cleavage sites of rat ProTα by active caspase-3, we performed MALDI-TOF analysis. As shown in Figure 6e, MALDI-TOF analysis revealed that the molecular mass of purified ProTα in naive C6 glioma cells was 12259.99, which corresponds to N-terminal serine acetylated full-length size (Ac2-112), whereas ProTα-like molecule in apoptogenic staurosporine-treated cells was 10480.39 or 10596.43, which corresponds to C-terminal truncated ProTα Ac2-97 or Ac2-98, respectively. Tunicamycin or etoposide treatments also showed similar results (data not shown). Next, we tried to characterize the cleavage region of recombinant rat ProTα (rrProTα) by caspase-3 by use of peptide mass fingerprinting analysis. In this experiment, rrProTα was incubated with or without active rat caspase-3, followed by a separation with SDS-PAGE, in-gel digestion of protein bands with trypsin and MALDI-TOF analysis (Figure 6f). In the absence of incubation of active rat capase-3 (blue peaks), two peaks with molecular mass of 1131.15 and 1479.66 were identified as a.a.22–31 and a.a.91–103 peptides, respectively. However, in its presence (black peaks), there were new peaks with 792.47 and 907.41, corresponding to a.a.91–97 and a.a.91–98 peptides, respectively, but the peak with 1479.66 disappeared. Furthermore, the digestion of synthetic rat ProTα C-terminal 93–112 polypeptide by caspase-3 produced two peaks with molecular mass of 1678.88 and 1794.01, which correspond to a.a.99–112 and a.a.98–112 peptides, respectively (Figure 6g). All these results indicate that rat ProTα has the two over-lapping cleavage sites by caspase-3 (Figure 6d). Accordingly, ProTα likely loses its NLS moiety under apoptotic conditions. This view is supported by the finding that immunoreactive ProTα is no longer localized in the nucleus after treatment of cells with apoptogenic compounds (Figure 6a). On the basis of the finding that the ProTα fragment was not released from cells, this C-terminal region appears to be essential for the extracellular release of ProTα.

Discussion

The nuclear protein ProTα currently attracts the interest of investigators in terms of life and death decisions in various cell types. In the nucleus, this protein epigenetically stimulates cell proliferation by binding to histones,[22] p300 histone acetyl-transferase,[23] and CREB-binding protein.[24] ProTα also enhances estrogen receptor transcriptional activity by binding to its repressor,[25] and binds to Keap1 to release Nrf2, which in turn upregulates various kinds of anti-oxidant enzymes important for survival.[26] Under the apoptotic conditions, it inhibits apoptosome formation.[4] Most recently, we discovered this protein as an anti-necrosis factor in the CM of cultured cortical neurons under serum-free starving conditions causing

Figure 5 S100A13 is a cargo molecule mediating extracellular ProTα release. **(a)** S100A13 Δ88–98 mutant as a dominant-negative regulator of ProTα release. Distributions of ProTα and *Strep*-tagII-S100A13 mutants in cultured C6 glioma cells with or without serum. **(b)** Loss of interaction of the *Strep*-tagII-S100A13 Δ88–98 mutant with ProTα in living cells. Cells and CM from C6 glioma cells stably expressing *Strep*-tagII-S100A13 mutants with or without serum (3 h) were harvested and a *Strep*-tagII pull-down assay was performed. Re: recombinant rat proteins. **(c)** The interaction of the *Strep*-tagII-S100A13 with ProTα occurs in cytosol, but not nucleus. Nucleus (N) and cytosol (C) from C6 glioma cells stably expressing *Strep*-tagII-S100A13 mutants with or without serum (3 h) were prepared and a *Strep*-tagII pull-down assay was performed. **(d–f)** Imaging of the ProTα–S100A13 interaction in living cells. Time-course of FRET images after serum-deprivation stress in phenol red-free DMEM. DsRed2-S100A13 was transiently expressed in C6 glioma cells stably expressing ProTα-EGFP. The FRET ratio represents the emission ratio of 590/520 nm. **(d)** Serum-deprivation stress-induced increase in the FRET emission ratio. Cell population images of FRET were measured in the presence of amlexanox (100 μM). Serum-deprivation stress caused an increase in the emission ratio from approximately 90 min. When the acceptor (DsRed2-S100A13) was earlier bleached, serum-deprivation stress did not increase the emission ratio. In cells expressing EGFP instead of ProTα-EGFP, FRET did not occur after serum deprivation. **(e)** Inhibition of FRET by the ProTα C-terminal peptide. Cellular delivery of a C-terminal peptide (amino-acids 102–112; forward peptide) of ProTα abolished increase of FRET emission ratio and decreased basal level, whereas no change was observed with the reverse peptide. **(f)** Inhibition of FRET by Ca^{2+} chelating. Cytosolic and extracellular Ca^{2+}-chelating agents, BAPTA-AM, and EGTA, respectively, inhibited FRET after serum deprivation. A decrease in the basal level of the emission ratio was observed approximately 70 min after the stress, suggesting that nuclear ProTα-EGFP is redistributed into the cytosol, but does not interact with S100A13 in the absence of Ca^{2+}. BAPTA-AM and EGTA were used at 1 μM and 1 mM, respectively. **(g)** Subcellular distribution of ProTα-EGFP and DsRed2-S100A13. The images were collected 180 min after serum deprivation after FRET analysis. **(h)** Inhibition of extracellular release of ProTα-EGFP and DsRed2-S100A13. The images were collected 180 min after serum-deprivation stress. Scale bars represent 20 μm

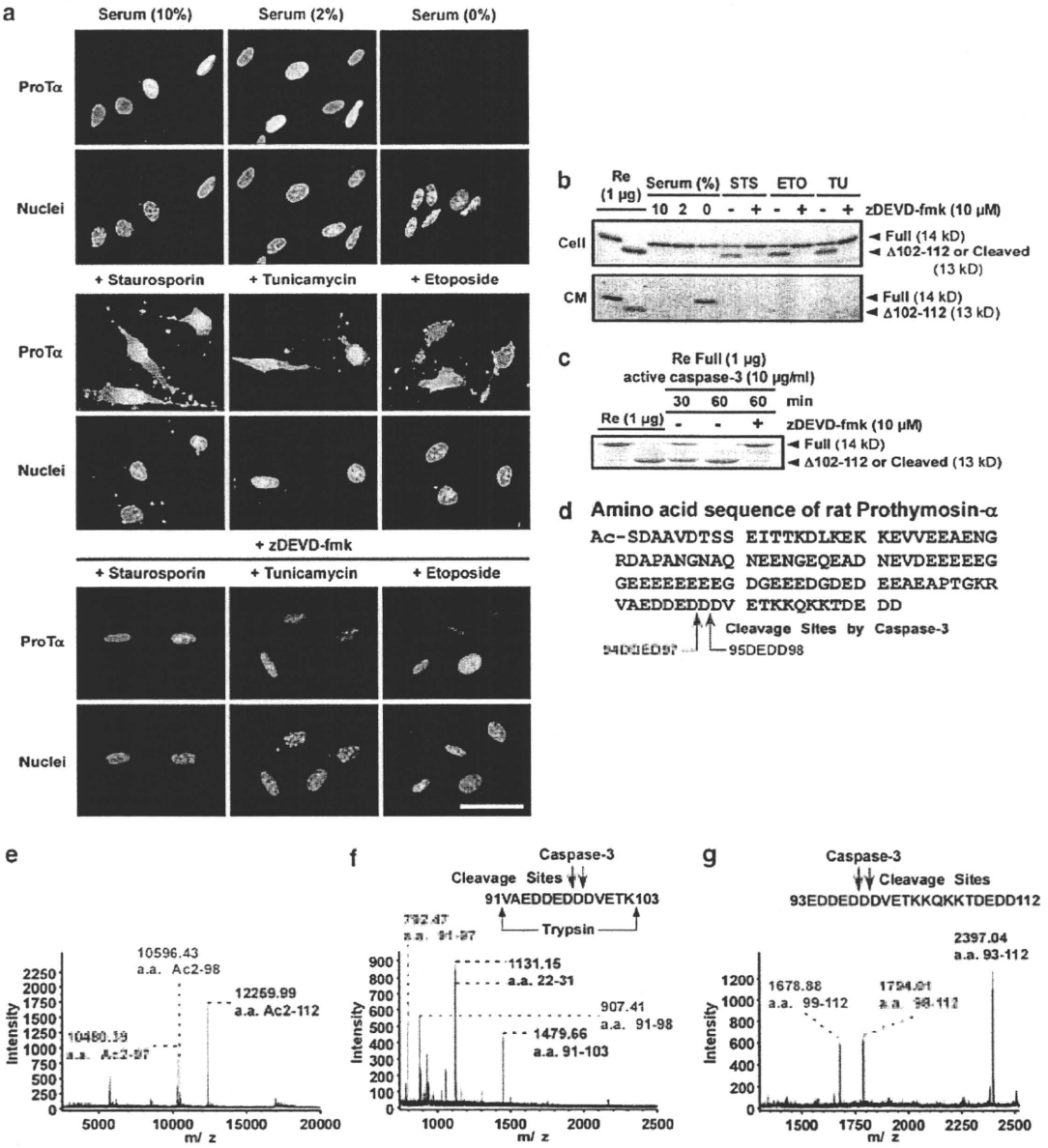


Figure 6 Apoptotic stress induces re-distribution of ProTα. (**a**) Altered distribution of ProTα in C6 glioma cells. Cells were subjected to serum-reduced (2%) or serum-deprivation stress for 3 h. Cells were treated with staurosporine, tunicamycin, or etoposide for 3 h in the presence or absence of zDEVD-fmk (10 μM). All apoptogenic compounds were used at 1 μM in 2% serum-containing medium. Scale bars represent 20 μm. (**b**) Cleavage, but not release of ProTα by apoptogenic compounds treatment. STS, staurosporine; TU, tunicamycin; ETO, etoposide. (**c**) Rat ProTα as a caspase-3 substrate. Recombinant (Re) rat ProTα (1 μg) was incubated with 10 μg/ml recombinant active caspase-3 in the presence or absence of 10 μM zDEVD-fmk for 1 h at 37°C. Recombinant rat ProTα Δ102–112 mutant was used as a marker of ProTα fragment lacking the C-terminal region. (**d**) Cleavage sites of rat ProTα by active caspase-3. (**e–g**) Identification of cleavage sites of rat ProTα by active caspase-3 by use of MALDI-TOF analysis. (**e**) Generation of two truncated types of ProTα by apoptogenic compound treatment. Peaks of intracellular native ProTα and cleaved ProTα by staurosporine treatment were shown as blue and black, respectively. The molecular mass of 12259.99 represents N-terminal serine acetylated full-length size (Ac2-112). The molecular mass of 10480.39 or 10596.43, which corresponds to C-terminal truncated ProTα Ac2-97 or Ac2-98, respectively. (**f**) Characterization of cleavage of ProTα by caspase-3 by peptide mass fingerprinting. Recombinant rat ProTα was incubated with or without active rat caspase-3, followed by a separation with SDS-PAGE, in-gel digestion of protein bands with trypsin and MALDI-TOF analysis. In the absence of incubation of active rat capase-3 (blue peaks), two peaks with molecular mass of 1131.15 and 1479.66 were identified as a.a.22–31 and a.a.91–103 peptides, respectively. However, in its presence (black peaks), there were new peaks with 792.47 and 907.41, corresponding to a.a.91–97 and a.a.91–98 peptides, respectively, but the peak with 1479.66 disappeared. (**g**) Cleavage of rat ProTα C-terminal regions by active caspase-3. Blue peak with molecular mass of 2397.04 represents synthetic rat ProTα C-terminal 93–112 polypeptide. The digestion of C-terminal polypeptide by caspase-3 produced two peaks with molecular mass of 1678.88 and 1794.01, which correspond to a.a.99–112 and a.a.98–112 peptides, respectively

necrosis.[5] Extracellular ProTα completely inhibits neuronal necrosis, but causes apoptosis in a different manner in cultured neurons.[27–29] However, after stroke, endogenous neurotrophins, such as brain-derived neurotrophic factor (BDNF) or erythropoietin, have been found to inhibit the apoptosis induced by exogenously administered ProTα.[6] In

the retinal ischemia model, the ischemic stress depletes ProTα from the retinal cells, and the intravitreous pretreatments with anti-ProTα IgG or antisense oligodeoxynucleotide against ProTα deteriorated the ischemic damage.[7] Therefore, it is evident that ProTα is extracellularly released from nuclei on ischemic stress, and that it exerts endogenous neuroprotective functions.

Polypeptide secretion has largely been defined as a process of exocytosis through the fusion of vesicles containing bioactive substances to the plasma membrane. In the representative vesicular secretion pathway, polypeptides that possess a signal peptide sequence in their N-terminal region are sorted to the ER-Golgi system to be processed by exocytosis.[30,31] However, several polypeptides possess extracellular functions despite lacking a signal sequence. The extracellular release of such polypeptides has to proceed through ER-Golgi-independent or non-vesicular (so-called non-classical) routes. It should be noted that these polypeptides showing non-classical extracellular release in general have significant functions in the life and death decisions of cells, as seen in the cases with angiogenic growth factors, inflammatory cytokines, extracellular matrix growth factors, viral proteins, and parasite surface proteins.[32,33] Unlike vesicular release, this type of release is caused by non-physiological stressful stimuli, which may cause rapid cell death. A series of pioneering studies by Maciag and his coworkers led to the hypothesis that S100A13 has key functions in the so-called non-classical extracellular release.[8] S100A13 has two EF-hand Ca^{2+}-binding motifs and belongs to a member of the S100 family.[15–17] It has been reported that S100A13 is involved in the non-classical extracellular release of target molecules containing fibroblast growth factor-1 (FGF-1) and interleukin-1α.[9–11,19,34,35] In this study, we successfully showed that ProTα is another example of stress-induced non-vesicular extracellular release, using S100A13, a cargo molecule. Furthermore, we revealed that the C-terminal regions of ProTα and S100A13 are essential for their interaction, which precedes extracellular release of both proteins, and that caspase-3 cleaves off C-terminal regions of ProTα. Thus, the non-vesicular extracellular release of ProTα has a unique feature that it is does not occur under the condition of apoptosis.

The unique point in this study is observed in the fact that ProTα is strictly localized in the nucleus in neurons, astrocytes, and C6 glioma cells. The mechanism underlying ischemic stress-induced extracellular release of ProTα comprises a two-tier export system: from the nucleus to the cytosol, and from the cytosol to outside the cell. As mentioned above, the nuclear export of ProTα is attributed to ischemia-induced ATP loss, which impairs the importin-NLS mechanism. As this nuclear export was not affected by leptomycin B, the serum-deprivation stress-induced drastic decrease in nuclear levels of ProTα is likely to be due to passive diffusion. Interestingly, once ProTα is exported from the nucleus, it is disappeared without remaining in the cytosol. In other words, nuclear export is the rate-limiting step for non-vesicular and extracellular release of ProTα.

The stress-induced extracellular release of ProTα was impaired by the addition of cytosolic or extracellular Ca^{2+}-chelating agents. The interaction between ProTα and S100A13 was Ca^{2+} concentration dependent in the range 0.1–200 μM, which corresponds to the cellular levels between resting and stimulated conditions. The Ca^{2+} dependency seems to be attributed to the facts that both S100A13 and ProTα are Ca^{2+}-binding proteins.[15–17,36] On the basis of the observation that serum-deprivation stress causes the activation of voltage-dependent N-type Ca^{2+} channel, which is involved in the non-classical release of FGF-1 and S100A13,[34,35] ischemic stress-induced Ca^{2+} influx may underlie the non-vesicular ProTα release as the second step after ATP loss-dependent nuclear release.

Neurons die by necrosis in the low density of culture under the serum-starved condition, but their survival activity increases as the cell density goes. We identified ProTα as the important molecule, which is released in the CM of serum-starved culture of neurons, and suppresses the necrosis through a recovery of glucose transport and ATP supply.[5,37] After longer culture with ProTα, however, we found that neurons die by apoptosis through activation of caspase-3.[5] As caspase-3 is known to cleave poly-[ADP-ribose] polymerase, which consumes abundant ATP molecule for the restoration from stress-induced damage of DNA,[38] this machinery seems to have some functions in suppression of rapid necrosis by stress.[29] As the concomitant addition of anti-apoptotic neurotrphins with ProTα completely inhibits the cell death, the physiological meaning of ProTα action would be speculated as a conversion of uncontrollable cell death necrosis to controllable apoptosis. Indeed, this speculation was confirmed by *in vivo* study, in which exogenous ProTα inhibited both necrosis and apoptosis of retinal cells after ischemia, but the further treatment with anti-BDNF antibody disclosed the apoptosis induction by ProTα.[7]

It should be noted that ProTα in the cytosol inhibits apoptosis through an inhibition of apoptosome formation in non-neuronal HeLa cells.[4] Furthermore, there is a report that ProTα is released from the nuclei when the NLS is cleaved off by caspase-3.[20,21] This study clearly showed that the C-terminal region of ProTα including NLS is cleaved in culture by the apoptosis-induced compound, and in cell-free digestion of recombinant ProTα by active caspase-3 (Figure 6d–g). As ProTα devoid of C-terminal region (a.a.98 or 99–112) is conceived to lose the activity of interaction with S100A13 (Figure 4d), it will remain in the cytosol without extracellular release. All these findings enable us to speculate that ProTα extracellularly released on the necrosis condition inhibits neuronal necrosis in an autocrine or paracrine manner, whereas cytosolic ProTα redistributed from the nuclei on the apoptotic condition may have an anti-apoptotic self-defensive function.

It is well known that several endogenous molecules are released in response to injury, infection, or other inflammatory stimuli, and initiate inflammatory responses. These molecules are so-called damage-associated molecular patterns (DAMPs) and/or alarmins.[39,40] Although DAMPs have intranuclear and/or intracellular functions under normal conditions, they have extracellular effects under pathological conditions. High-mobility group box1 (HMGB1), a representative DAMP molecule, has similar characteristics to ProTα. HMGB1, a nuclear protein, is released by necrotic stress, but not by apoptosis, as seen for ProTα. However, it causes