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Secretion and uptake of TAT-fusion proteins produced by engineered mammalian cells

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Abstract

Background—Intracellular signaling can be regulated by the exogenous addition of physiological protein inhibitors coupled to the TAT protein transduction domain. Thus far experiments have been performed with purified inhibitors added exogenously to cells *in vitro* or administered *in vivo*. Production of secretable TAT-fusion proteins by engineered mammalian cells, their uptake, and route of entry has not been thoroughly investigated. Such methodology, if established, could be useful for transplantation purposes.

Methods—Secretion of TAT-fusion proteins from transfected mammalian cells was achieved by means of a signal peptide. Cell uptake and subcellular localization of TAT-fusion proteins were determined by immunoblotting and confocal microscopy.

Results—Engineered TAT-fusion proteins were secreted with variable efficiency depending on the nature of the protein fused to the TAT peptide. Secreted proteins were able to transduce unmanipulated cells. Their mechanism of entry into cells partly involves lipid rafts and a portion of the internalised protein is directed to the Golgi.

Conclusions—Generation of secretable TAT-coupled inhibitors of signaling pathways, able to transduce other cells can be achieved.

General significance—These results provide key information that will assist in the design of TAT-inhibitors and engineered cells in order to regulate cell function within tissues.

Keywords

Protein transduction domain; TAT; Cell penetrating peptide; Lipid raft; Signaling inhibitor

1. Introduction

The term protein transduction domain (PTD) is used to define short cationic peptides that are either derived from proteins or are synthetic and which have the capacity to enter cells [1-3]. They are also known as cell-penetrating peptides (CPPs) [4]. These peptides can deliver heterologous proteins inside cells but also other types of cargo such as nucleic acids, peptides, viral and phage particles, and small chemical compounds (reviewed in [1,5]). One such commonly used PTD is derived from the HIV TAT protein (amino acids 48-60) [6,7]. Genetic fusion or chemical conjugation of the TAT PTD has assisted in delivering physiological protein inhibitors into cells to down-regulate signaling pathways both *in vitro* and *in vivo* (reviewed in [1,5]). We have used the TAT-mediated cell transduction to

introduce into cells the Inhibitor of κ B alpha ($\text{I}\kappa\text{B}\alpha$) protein. $\text{I}\kappa\text{B}\alpha$ is a physiological inhibitor of NF- κ B, a transcription factor with a crucial role in inflammation [8,9]. We genetically fused the TAT PTD to a mutated form of $\text{I}\kappa\text{B}\alpha$ which is a potent inhibitor of NF- κ B activity and is commonly referred to as super repressor ($\text{srI}\kappa\text{B}\alpha$) [10]. We have shown that purified TAT- $\text{srI}\kappa\text{B}\alpha$ was effective in reducing NF- κ B activity in cells *in vitro* [11] and in down-modulating the severity of inflammation when administered *in vivo* [12].

The vast majority of experiments described in the literature have been performed with purified TAT-proteins produced in bacteria [7]. The possibility of engineered mammalian cells producing a secretable TAT-fused inhibitor has not been adequately explored. A recent publication investigated how different PTDs, including variations of the prototypical TAT peptide, affect secretion of the CRE protein by mammalian cells and its subsequent uptake by target cells [13]. It was found that the charge of the PTD plays a role in the secretion efficiency, an observation that should be taken into account when designing secretable chimeras. In another report, TAT-GFP produced endogenously in mammalian cells transfected with the relevant plasmid, was found to accumulate predominantly in the nucleus and the nucleolus [14]. In this study we investigate whether the protein partner of the TAT PTD affects secretion of chimeric proteins. Importantly, we examine secretion and uptake of 2 physiological inhibitors of key signaling pathways and show that these chimeric proteins can be secreted but with variable efficiencies. Cells engineered to produce cell-transducing inhibitors could form the basis for cell transplantation-mediated therapy in certain pathological conditions where aberrant signaling underpins disease pathology. In the work presented here, using the EGFP reporter protein and the $\text{srI}\kappa\text{B}\alpha$ inhibitor fused to TAT peptide and to a secretion signal, we study production, secretion and uptake of TAT-fusion proteins *in vitro*. In addition, using confocal microscopy, we investigate cellular distribution of internalised TAT-fusion proteins.

2. Materials and methods

2.1. Expression constructs and transfection

Cloning of the human $\text{srI}\kappa\text{B}\alpha$ mutant gene into the prokaryotic expression vector pRSET-TAT-HA (a kind gift from S. Dowdy, UCSD, CA) has been described previously [11]. Using this construct as template the TAT-HA- $\text{srI}\kappa\text{B}\alpha$ -V5 region was amplified with PCR using the forward primer 5'-TCGATATCGTACGGCCGCAAGAAACGC-3' and the reverse primer 5'-CTGCTAGCCGGATCAAGCTTCGA-3'. The PCR product was digested with EcoRV/NheI and subcloned in frame into the corresponding sites of the commercial vector pFUSE-mFc2(IL2ss) (InvivoGen, San Diego, CA). This vector contains the 21 amino acid secretory signal (ss) from the IL2 gene upstream of the Fc portion of the mouse IgG heavy chain (FcIgG). The final construct generates an IL2ss-TAT-HA- $\text{srI}\kappa\text{B}\alpha$ -V5 polypeptide. To produce an IL2ss-TAT-HA-EGFP construct, the $\text{srI}\kappa\text{B}\alpha$ -V5 segment was excised using the internal KpnI/SphI sites [11] and replaced with the EGFP (enhanced GFP) gene, which was amplified by PCR using as template the commercial vector pEGFP-N2 (BD Biosciences, Palo Alto, CA) and primers 5'-ATTGGTACCGTGAGCAAGGGCGAGGAG-3' (forward) and 5'-GCTGCATGCTTACTTGTACAGCTCGTC-3' (reverse). Similarly the IL2ss-TAT-HA-RBD (RBD, Ras binding domain of Raf-1) construct was generated by replacing the FcIgG part of the original pFUSE vector with the PCR-amplified TAT-HA-RBD domain (from the pRSET-TAT-HA-RBD prokaryotic expression vector) and inserting it into the EcoRV/NheI cloning sites. Transfection of cells with the pEGFP-N2 vector which does not contain the secretion signal was used to assess non-specific leakage of protein from cells. In some experiments we used purified TAT-HA- $\text{srI}\kappa\text{B}\alpha$ -V5 and TAT-HA-GFP as positive controls for cell transduction. These proteins were produced in bacteria and purified as described before [7,11].

2.2. Cells, antibodies and other reagents

The 293T cell line was maintained in DMEM/10% foetal calf serum (FCS) medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine. The T cell line Jurkat was maintained in RPMI-1640/5% FCS plus the aforementioned supplements. Antibodies to IκBα and p65 NF-κB were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-GFP monoclonal antibody (mAb) was from Roche, anti-V5 from Invitrogen (Carlsbad, CA), anti-Giantin from Abcam (Cambridge, UK), and Alexa-594-conjugated donkey anti-rabbit and Alexa-488-conjugated anti-mouse from Molecular Probes (Eugene, OR). The DRAQ5™ dye for nuclear staining was from Biostatus Limited (Leicester, UK) and Alexa fluor 555-conjugated cholera toxin B subunit (CTB) from Molecular Probes (Eugene, OR).

2.3. Protein transduction, immunoprecipitation and Western blotting

To assess uptake of secretable TAT-fusion proteins, Jurkat cells were incubated at 37 °C for 2-4 h with the indicated supernatant collected from cultures of transfected cells, concentrated and filtered to remove any cell debris. Following incubation, cells were washed with PBS and treated with trypsin for 10 min at 37 °C to digest membrane-attached TAT-fusion proteins, and lysed in 1% NP-40-containing buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40) supplemented with a protease inhibitor cocktail (Leupeptin, Pepstatin A, E-64, Bestatin, Aprotinin and AEBSF hydrochloride, from Calbiochem, La Jolla, CA), and phosphatase inhibitors (10 mM of sodium orthovanadate from Sigma-Aldrich, Dorset, UK and 0.3 µM okadaic acid from Calbiochem). Cell lysates were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (PVDF) and Western blotted with the indicated antibodies. Horseradish peroxidase (HRP)-conjugated secondary Abs and ECL (Amersham Biosciences, Uppsala, Sweden) were used to develop the Western blots.

Immunoprecipitation of p65 NF-κB from 293T cells transfected with the pFUSE-TAT-srIκBα or the pFUSE control, was performed by addition of 5 µg of an agarose-conjugated anti-p65 antibody (Santa Cruz) to pre-cleared cell lysates. Immune complexes were resolved by 10% SDS-PAGE and immunoblotted sequentially with anti-V5 and anti-p65 antibodies.

2.4. Intracellular staining and confocal microscopy

Jurkat T cells were incubated with the indicated supernatants for 4 h at 37 °C, treated with trypsin for 10 min to eliminate membrane bound protein, washed extensively and fixed in 4% paraformaldehyde (PFA). After fixation cells were permeabilised with 0.5% TritonX-100, blocked with 0.5% fish skin gelatin for 1 h and incubated with anti-GFP or anti-V5 antibodies. Following staining the cells were attached onto poly-L-lysine-treated slides (Sigma-Aldrich, Dorset, UK), mounted with CitiFluor AF1 solution (CitiFluor Ltd, London, UK) and analysed using the Zeiss LSM510META confocal microscope (Jena, Germany). Images were acquired using a 100× oil immersion objective and the LSMS10Meta software after adjusting to the same output intensities. To determine subcellular colocalisation, treated cells were either double stained with antibodies to the indicated TAT-protein and CTB-Alexa 555, or triple stained with anti-TAT-protein, anti-Giantin, and the DRAQ5™ dye.

3. Results

3.1. TAT-chimeric proteins are secreted with variable efficiency

To develop secretable TAT-fusion proteins we subcloned 3 different polypeptides into the pFUSE-mFc2 vector which contains the secretion signal from the IL2 gene. All polypeptides incorporate the TAT PTD downstream of the secretion signal. The proteins used are the

EGFP reporter, the $\text{srI}\kappa\text{B}\alpha$ inhibitor of $\text{NF-}\kappa\text{B}$, and the Ras inhibitor RBD. A schematic depiction of the 3 chimeras and the secreted FcIgG is shown in Fig. 1A.

To assess the ability of the various TAT-fusion proteins to be secreted, 293T cells were transiently transfected with the expression constructs. 24 or 48 h post-transfection, the cell culture supernatants were collected (5 ml total volume) and cells were washed and lysed with 500 μl lysis buffer. Aliquots of cell culture supernatant and cell lysate from each transfection representing 1/100 of total were resolved by SDS-PAGE and the level of TAT-proteins was determined by immunoblotting (Fig. 1B). Supernatants were filtered before manipulation to eliminate any cell debris. All TAT-fusion proteins were present in supernatants from transfected cultures. We have noticed 2 distinct protein bands in both lysates and supernatants from pFUSE-TAT-EGFP transfected cells, migrating above the 25 kDa marker. Multiple bands (at least 3) were also seen in pFUSE-TAT- $\text{srI}\kappa\text{B}\alpha$ transfected cells (Fig. 1B and C). It is unclear at present why these constructs produce multiple bands on SDS-PAGE.

Although a substantial amount of the produced FcIgG was found in the supernatant (approximately 50% of total), secretion of the chimeric proteins was much lower (Fig. 1B). Levels of secretion also differed between the 3 TAT-chimeras with the TAT- $\text{srI}\kappa\text{B}\alpha$ being secreted at lower levels compared to TAT-EGFP and TAT-RBD (Fig. 1B). Nevertheless, no EGFP was detected in the supernatant from cells transfected with a vector expressing EGFP without the secretion signal excluding the possibility of non-specific leakage of proteins into the supernatant (Fig. 1B, bottom panel). Therefore, intracellular proteins can be secreted when placed under the control of an appropriate secretion signal however secretion efficiency is considerably lower when compared to a naturally secreted protein. In order to increase concentration in subsequent experiments we used 10 $\times$ concentrated supernatants with the exception of FcIgG-containing supernatant which was used unconcentrated (Fig. 1C).

To see if one reason behind the lower secretion of TAT- $\text{srI}\kappa\text{B}\alpha$ is association with its natural partner, p65 $\text{NF-}\kappa\text{B}$ was immunoprecipitated from lysates of cells transfected with either pFUSE-TAT- $\text{srI}\kappa\text{B}\alpha$ or FcIgG control, and analysed for the presence of co-immunoprecipitating TAT- $\text{srI}\kappa\text{B}\alpha$. As seen in Fig. 1D, TAT- $\text{srI}\kappa\text{B}\alpha$ can associate with endogenous $\text{NF-}\kappa\text{B}$ and this interaction might anchor the chimeric protein inside the producing cell. Lack of p65 presence in control antibody immunoprecipitation confirmed the specificity of the anti-p65 reactivity (Fig. 1E).

3.2. Cell transduction by secreted TAT-fusion proteins

To assess if the secreted TAT-fusion proteins can transduce unmanipulated cells, Jurkat T cells were incubated with concentrated supernatant from pFUSE-TAT-EGFP transfections. Incubations were carried out at 37 $^{\circ}\text{C}$ for 2 and 4 h, with a 5 $\times$ diluted supernatant for 4 h, or with supernatant from pFUSE vector-transfected cells as control. After incubation cells were treated with trypsin to eliminate surface-bound protein, lysed and analysed for the presence of internalised TAT-EGFP. Two protein bands migrating above the 25 kDa marker were detected in anti-GFP immunoblots (Fig. 2A, bottom panel), the size of which correlated with the size of the TAT-EGFP bands detected in the concentrated supernatants (Fig. 2A, top panel). The intensity of the 2 bands was significantly reduced in cells incubated with 5 $\times$ diluted supernatant (Fig. 2A, bottom panel). Therefore, secreted TAT-EGFP can transduce cells and its levels are proportional to its concentration in the supernatant. Fig. 2B shows that the trypsin treatment was able to eliminate any secretable fusion protein in supernatants.

Next Jurkat cells were incubated with TAT- $\text{srI}\kappa\text{B}\alpha$ -rich supernatant or with TAT- $\text{srI}\kappa\text{B}\alpha$ purified from bacteria [11] as positive control for cell transduction. As mentioned above the

secreted TAT-srI κ B α is a mixture of at least 3 protein species, while the purified TAT-srI κ B α which contains additional sequences at the N-terminus migrates at approximately 50 kDa on SDS-PAGE (Fig. 2C). As expected, purified TAT-srI κ B α transduced cells as indicated by the appearance of a 50 kDa band in anti-V5 western blots. Similarly, anti-V5 blots of lysates from cells treated with TAT-srI κ B α -rich, but not control, supernatant revealed a reactive band migrating above the 37 kDa marker (Fig. 2C). This band corresponds to the smaller of the species seen in supernatants from transfected cells and its size is closer to the theoretical size estimated according to the primary structure of the chimera. The nature of the larger bands in the supernatant which fail to transduce cells is unknown. One possibility is that following cell transduction, the secreted protein from mammalian cells is more prone to cleavage compared to *E. coli*-produced protein.

3.3. Subcellular localisation of internalised TAT-proteins

To investigate the subcellular location of TAT-fusion proteins after cell transduction, Jurkat cells were incubated with concentrated supernatants containing TAT-EGFP or purified TAT-GFP produced in bacteria, stained with anti-GFP antibodies and analysed by confocal microscopy. Cells treated with supernatant from non-transfected cells and stained with anti-GFP antibodies served as negative control. Fig. 3A shows that most of the internalised protein was located in the cytoplasm and had a rather punctated staining pattern. The nucleus of the cells remained by enlarge free of transducing protein. Similar experiments were performed with purified TAT-srI κ B α or supernatant containing TAT-srI κ B α . Anti-V5 staining of treated Jurkat cells showed that internalised TAT-srI κ B α mostly concentrates in defined areas in the cytoplasm (Fig. 3B). The pattern of staining was more punctuated compared to TAT-EGFP and most of the fluorescence was concentrated in structures located in one side of the cell rather than throughout the cytoplasm. Frequently TAT-srI κ B α was seen concentrating in a defined structure adjoining the nucleus (Fig. 3B last panel). In some cells nuclear localisation of internalised TAT-srI κ B α was also visible (Fig. 4, middle panel). Internalisation of TAT-EGFP was also visible in live, unfixed cells that were incubated with TAT-EGFP-rich supernatant for 4 h (Fig. 3C), a result that excludes the possibility that the observed internalisation is due to a fixation artefact. However, differences in the staining pattern were noted between fixed (Fig. 3A) and unfixed (Fig. 3C) cells, suggesting that the fixation step has an effect on the localization of TAT-EGFP.

To obtain more information about the cellular structures to which internalised TAT-proteins are concentrating and to gain information regarding the mechanism of entry, we performed double and triple staining of treated cells. It has been shown recently that TAT PTD-containing proteins are internalised via caveolae/lipid raft-mediated endocytosis [15-17]. To assess whether the secretable TAT-srI κ B α inhibitor in our system uses lipid rafts to enter cells, Jurkat cells were treated with control or TAT-srI κ B α -rich supernatant or with purified TAT-srI κ B α and double stained with anti-V5 antibodies and CTB which binds to the lipid raft marker GM1. As shown in Fig. 4, the structures/vesicles which concentrate most of the internalised TAT-srI κ B α also stained for the lipid raft marker GM1 indicating colocalisation.

To see if the structure adjoining the nucleus is the Golgi apparatus, treated cells were triple stained with an antibody to Giantin, a marker for Golgi, plus anti-GFP or anti-V5 antibodies. A DNA-binding dye was used to reveal the cell nucleus. As mentioned above, internalised TAT-GFP was present in the cytoplasm and a portion of the protein localised to the Golgi as shown by its colocalisation with anti-Giantin antibodies (Fig. 5A). In the case of TAT-srI κ B α -treated cells most of the internalised protein was concentrated in the Golgi apparatus as shown by the merging of the anti-V5 and anti-Giantin images (Fig. 5B). These results suggest that lipid rafts may play a role in the internalisation of TAT-proteins and that part of

the internalised protein concentrates in the Golgi, although the levels may vary depending on the nature of the transducing protein.

4. Discussion

For certain diseases cell therapy could become a viable and economical option for their treatment. A group of such diseases are those characterised by chronic unresolved tissue inflammation. In the last few years the PTD-mediated protein transduction has opened new avenues for the intracellular delivery of protein and peptide inhibitors in order to control cell function. A practical outcome of the PTD studies has been the delivery of physiological inhibitors of key signal transduction cascades and regulation of their activity *in vitro* and *in vivo*. We have concentrated on the NF- κ B transcription factor pathway because it controls inflammation [11,12]. In previous experiments the TAT-coupled inhibitor was produced and purified in a prokaryotic expression system. However, with this protocol, a continuous supply of purified inhibitor will be needed to control chronic inflammation in a sustained way. It is possible that this drawback could be overcome by transplantation of cells engineered to produce a secretable form of the inhibitor within the context of the inflamed tissue. If the secreted inhibitor can be produced in sufficient amounts and taken up by surrounding cells, then local down-modulation of inflammation may be achievable. An additional advantage of such methodology will be that, in contrast to the systemic administration, healthy tissues will not be exposed to the TAT-coupled inhibitor thus reducing potential side effects. To achieve this however, a number of fundamental questions have to be answered. For example, it is unknown if a secretable TAT-coupled inhibitor can be generated and secreted efficiently from mammalian cells, or if it will be capable of transducing other cells. The results presented here begin to address such questions. We have generated 3 different secretable TAT-fused polypeptides, 2 inhibitors and a reporter protein. By comparing secretion efficiency of these polypeptides to a naturally secreted protein, we found that although proteins such as I κ B α and RBD, which are normally intracellular polypeptides, can be secreted, the efficiency of secretion is low compared to FcIgG (Fig. 1). Furthermore, our results suggest that the protein partner of the TAT-PTD chimera can influence secretion. We found that TAT-srI κ B α associates with endogenous NF- κ B in producing cells, suggesting that a reason for its low secretion could be its retention inside the cell (Fig. 1D). This raises the possibility that TAT-srI κ B α may act as an inhibitor in producing cells leading to changes in biological function. For example, in certain cell types, particularly immune cells, stimulation with pro-inflammatory mediators leads to apoptosis if NF- κ B activity is inhibited [18].

A recent relevant study tested different PTDs, the majority of them derived from modification of the original TAT PTD, for their capacity to deliver into cells a secreted CRE protein [13]. This study found that the positive charge of the PTD affects secretion. Instead in our study we tested secretion of different proteins while the PTD was kept the same for all constructs. Importantly, we tested secretion of physiological inhibitors of signaling cascades which would be relevant to therapy. Another report showed that when a TAT-fused β -glucuronidase, a lysosomal enzyme deficient in mucopolysaccharidosis VII disease, was expressed *in vivo*, enzyme activity was detected beyond the expressing cells indicating that the TAT PTD enhances the distribution of the enzyme within the tissue [19]. However, another group reported lack of intercellular transfer of TAT-GFP protein produced *de novo* [20]. An explanation for this result could be inadequate production of the TAT-fusion protein.

An important question currently addressed by a number of groups is to understand how PTDs enter cells. Initial reports suggested that their mechanism of entry is endocytosis-independent because PTDs could be seen inside cells even in the presence of ATP-depleting

agents [21,22]. Subsequently, however, this theory was discounted because of imaging artefacts which were created under certain conditions of cell fixation [23]. Such artefacts led to the erroneous conclusion that PTDs primarily accumulate into the nucleus upon entry. A possible explanation could be that excess PTD attaching to the cell membrane is redistributed to negatively charged structures inside cells, such as DNA, following fixation, giving the impression of strong nuclear localization. Treatment of cells with trypsin or heparin to digest or dislodge membrane attached proteins before fixation, have been used as a way to eliminate non-specific interactions [23]. In our experiments, to safeguard against imaging artefacts, we have treated cells with trypsin after incubation with TAT-fusion proteins or supernatants. We also visualize TAT-EGFP internalisation without anti-GFP antibody immunostaining (Fig. 3C).

The current model of internalisation proposes the initial binding of the positively charged PTDs to heparan sulphate proteoglycans on the cell surface [22,24,25]. Subsequently, entry of PTDs and their associated cargo into cells most likely occurs through energy-dependent endocytosis [23,26]. Lipid raft and caveolae are reported to mediate, at least in part, endocytosis of PTDs [15-17]. Clathrin-mediated endocytosis has also been reported [27]. What happens to the endocytosed material remains largely unknown. We have previously shown that internalised TAT-srI κ B α can associate with endogenous NF- κ B in transduced cells [11]. However, most likely only a portion of internalised TAT-srI κ B α escapes from endocytic vesicles to find its target. Furthermore, different PTDs or PTD-coupled proteins may be targeted to different cell compartments. As we report here the nature of the protein partner of the TAT PTD plays a role in determining localization. Therefore, generalizations should be avoided and endocytosis and subcellular localization of individual PTD-fusion proteins should be assessed independently.

Here by studying cell entry of secretable TAT-srI κ B α inhibitor and TAT-EGFP, we found that most of the internalised protein concentrates in vesicular structures with the majority of TAT-srI κ B α concentrating in a distinct area of the cell adjoining the nucleus (Fig. 3). Confocal imaging showed partial colocalisation between GM1, a marker for lipid rafts, and internalised TAT-srI κ B α . This result suggests that secretable TAT-srI κ B α uses a mechanism for cell entry similar to purified TAT PTD. It has been reported that uptake of TAT PTD by HeLa cells involves the Golgi apparatus. Bodipy ceramide, a Golgi marker, was shown to partially colocalise with labelled TAT PTD [28]. The finding in our studies that secretable TAT-srI κ B α concentrates in a distinct vesicular structure adjoining the nucleus led us to investigate if this structure overlaps with Golgi. Using a specific antibody to Giantin to visualize the Golgi, we found that there was substantial localisation of internalised TAT-srI κ B α , and to a lesser degree TAT-EGFP (Fig. 5). These results suggest that following their binding to the cell surface, secreted TAT-fusion proteins may use a retrograde mechanism of transport to the Golgi. The fate of internalised proteins after the Golgi remains unknown and it could depend on the nature of the protein partner of TAT PTD. It is worth noting however, that some nuclear localisation of TAT-srI κ B α has been seen during the course of these experiments (Fig. 4, middle panel). In summary, the results presented here add to our understanding of the process of cell entry of TAT-fusion proteins and should be taken into consideration when designing protein inhibitors of signaling pathways.

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Fig. 1.

Expression and secretion of TAT-fusion proteins from transfected cells. (A) Schematic depiction of the TAT-fusion proteins engineered to be secreted from expressing cells. ss: secretion signal; FcIgG: Fc portion of mouse IgG heavy chain. (B) 293T cells transfected with the indicated constructs were cultured for 48 h in 10 cm dishes after which the supernatants (5 ml total volume) were collected and the cells lysed in 500 μ l lysis buffer. Aliquots of lysates and supernatants (1/100 of total) were western blotted with the indicated antibodies. In the bottom panel, EGFP denotes transfection of cells with a vector that expresses EGFP without the secretion signal and treated identically to the pFUSE construct transfections. All transfections were performed in 10 cm culture dishes. (C) Western blot analysis of secreted proteins in concentrated spent supernatants from transfected cells. (D) Anti-V5 immunoblot (top panel) of p65 immunoprecipitation from lysates of cells transfected with TAT-srIkB α or FcIgG-expressing constructs. Levels of p65 NF- κ B immunoprecipitated were analysed by anti-p65 antibodies (bottom panel). Sizes of molecular weight markers migrated in the same gel are shown on the left. (E) Anti-p65 immunoblot of immunoprecipitations performed with control and anti-p65 antibody.

Fig. 2.

Cell transduction by secreted TAT-fusion proteins. (A) Jurkat T cells were cultured with concentrated supernatant from TAT-EGFP secreting cultures for the times shown. FcIgG containing supernatants were used as control. Aliquots from supernatants (top panel) and cell lysates (bottom panel) were probed with anti-GFP. (B) Culture supernatant from TAT-EGFP transfected cells was either treated with trypsin for 10 min or left untreated and then aliquots loaded onto gel were Western blotted with anti-GFP antibodies. (C) Jurkat cells were incubated for 4 h with TAT-srIκBα-containing supernatants or with purified TAT-srIκBα as positive control for cell transduction and aliquots of lysates and supernatants were probed with anti-V5 antibodies.

Fig. 3.

Subcellular localisation of internalised TAT-fusion proteins. (A) Jurkat cells were incubated with control supernatant, purified TAT-GFP, or supernatant containing secreted TAT-EGFP for 4 h at 37 °C. Following incubation cells were fixed, permeabilized and stained with anti-GFP antibodies. Background fluorescence was set by staining control supernatant-treated cells with anti-GFP antibodies. (B) Jurkat cells were incubated with control supernatant, purified TAT-srlkBα or supernatants containing secreted TAT-srlkBα and stained with anti-V5 antibodies. Background fluorescence was set by anti-V5 stain of cells incubated with control supernatant. 2 representative cells from each treatment are presented. (C) Jurkat cells were incubated for 4 h with concentrated TAT-EGFP-rich or control supernatant, treated briefly with trypsin, washed and then immediately imaged by confocal microscopy. Images were taken using a 100× objective. Bar=10 μm.

Fig. 4.

Colocalization of internalised TAT-srI κ B α with a lipid raft marker. Jurkat cells were cultured with control supernatant, purified TAT-srI κ B α , or supernatant containing secreted TAT-srI κ B α . Following incubation cells were fixed, permeabilised and double stained with anti-V5/anti-mouse-Alexa 488 (green) and CTB-Alexa-555 (red). Colocalization of green and red fluorescence is indicated by the development of yellow pseudo-colour in merged images. Bar=10 μ m.

Fig. 5.

Localisation of internalised TAT-fusion proteins to the Golgi. (A) Jurkat cells were treated with TAT-EGFP-rich supernatant, purified TAT-GFP, or control supernatant and triple stained with anti-GFP (green), anti-Giantin (red) and a DNA-binding dye (blue) to visualize internalised TAT-GFP, Golgi and nucleus respectively. (B) Jurkat cells were cultured with control or TAT-srIκBα-containing supernatant, or with purified TAT-srIκBα and immunostained with anti-V5 (green) and anti-Giantin (red) antibodies and with the DNA-binding dye (blue). Development of yellow pseudo-colour indicates colocalization of TAT-fusion protein with the Golgi marker Giantin. Bar=10 μm.