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Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector

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A fusion protein of acidic fibroblast growth factor and diphtheria toxin A-fragment was disulfide-linked to the toxin B-fragment. The complex bound specifically to diphtheria toxin receptors, and subsequent exposure to low pH induced the fusion protein to translocate to the cytosol. Heparin, inositol hexaphosphate and inorganic sulfate strongly increased the trypsin resistance of the growth factor part of the fusion protein, indicating tight folding, and they prevented translocation of the fusion protein to the cytosol. The data indicate that only a more disordered form of the growth factor is translocation competent.

Key words: acidic fibroblast growth factor/diphtheria toxin/membrane translocation

Introduction

Transport of proteins into and across cellular membranes is fundamental in biology, and in recent years the study of this process has become a rapidly developing field of research. In eukaryotic cells, protein translocation usually occurs from the cytosol into different cellular organelles, such as the endoplasmic reticulum, mitochondria, chloroplasts and peroxisomes (Wickner and Lodish, 1985; Walter and Lingappa, 1986; Imanaka *et al.*, 1987; Schatz, 1987; Pain *et al.*, 1988; Pon *et al.*, 1989; Hartl and Neupert, 1990). However, a number of toxic proteins from bacteria and plants can be translocated in the opposite direction and enter the cytosol from extracytosolic locations (Olsnes *et al.*, 1988).

The best studied example is diphtheria toxin, which is produced by *Corynebacterium diphtheriae* as a single polypeptide with a molecular weight of 58 kDa. Treatment with low concentrations of trypsin cleaves ('nicks') the toxin into two fragments, the N-terminal 21 kDa A-fragment and the C-terminal 37 kDa B-fragment (Pappenheimer, 1977; Gill, 1978). The B-fragment binds to cell surface receptors and facilitates membrane translocation of the A-fragment to the cytosol where it ADP-ribosylates elongation factor 2 and thereby inhibits protein synthesis and kills the cells (Collier, 1967; Honjo *et al.*, 1971; van Ness *et al.*, 1980).

The normal entry pathway for diphtheria toxin involves receptor-mediated endocytosis of the toxin from coated pits and subsequent translocation of the A-fragment across the limiting membrane of the endosome by a mechanism requiring low pH (Draper and Simon, 1980; Sandvig and Olsnes, 1980, 1981). This process can be mimicked at the level of the plasma membrane when cells with surface-bound nicked toxin are exposed to low pH (Moskaug *et al.*, 1988). Under these conditions the toxin undergoes a conformational change, the B-fragment is inserted into the membrane and the A-fragment is translocated to the cytosol. We have shown earlier that additional peptides at the N-terminal end of the A-fragment can be translocated to the cytosol along with the A-fragment (Stenmark *et al.*, 1991).

There is at present ample evidence that translocation of proteins from the cytosol to the endoplasmic reticulum, to mitochondria and to other organelles largely occurs with the protein in an unfolded form (Sanz and Meyer, 1988; Verner and Lemire, 1989; Watanabe and Blobel, 1989; Pfanner *et al.*, 1990; Rassow *et al.*, 1990). Binding of metothrexate to dihydrofolate reductase induced a more compact conformation of the protein and prevented its translocation into mitochondria (Eilers and Schatz, 1986). To test if this is also the case with diphtheria toxin-guided translocation, we have in the present paper studied whether a truncated form of acidic fibroblast growth factor (designated as aFGF

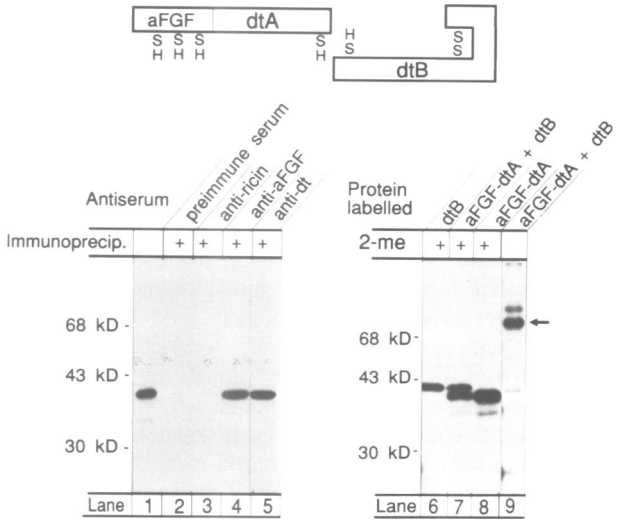

Fig. 1. Characterization of aFGF-dtA and its ability to form disulfide bonds with dtB. **Top**: Schematic presentation of aFGF-dtA and dtB. **Left**: pHBFG-dt1 was linearized with *Eco*RI, and mRNA encoding aFGF-dtA was transcribed with T3 RNA polymerase. The mRNA was translated in a reticulocyte lysate in the presence of [^{35}S]methionine. Lane 1, 1 μl translation mixture as such; lanes 2–5, 1 μl translation mixture was added to 25 μl protein A–Sepharose that had been preincubated with 2 μl preimmune serum (lane 2); 2 μl anti-ricin (lane 3), 2 μl anti-aFGF (lane 4) or 2 μl anti-diphtheria toxin (lane 5). The adsorbed material was eluted with SDS and analyzed by SDS–PAGE and fluorography. **Right**: Lane 6, 1 μl translation mixture containing [^{35}S]methionine-labelled dtB; lane 7, same as lane 9, but treated with 2-mercaptoethanol; lane 8, 1 μl translation mixture containing [^{35}S]methionine-labelled aFGF-dtA; lane 9, 1 μl of a mixture of lysates containing [^{35}S]methionine-labelled aFGF-dtA and dtB that had been dialyzed to remove reducing agents.

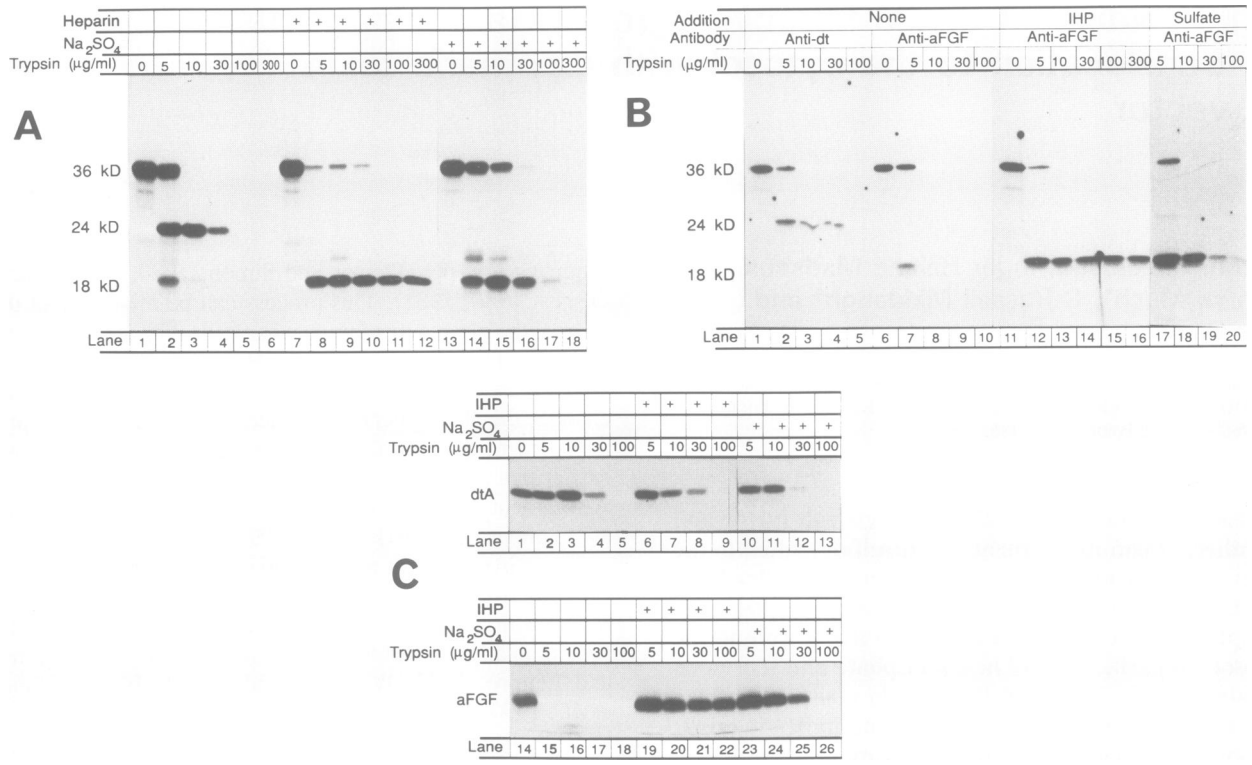

Fig. 2. Effect of heparin, inositol hexaphosphate and inorganic sulfate on the trypsin sensitivity of [aFGF-dtA-SS-dtB], dtA and aFGF. (A and B) Reticulocyte lysate containing [^{35}S]methionine-labelled aFGF-dtA was mixed with unlabelled dtB and dialyzed against HEPES medium, pH 7.4; 1 μl samples were then incubated in 10 μl HEPES medium, pH 4.5, with increasing concentrations of TPCK-treated trypsin for 1 h at 37°C. Then phenylmethyl sulfonyl fluoride (1 mM) was added. Panel **A**: The samples were analyzed by SDS−PAGE under reducing conditions before fluorography. Before trypsin treatment, the following compounds were added: lanes 1−6, no addition; lanes 7−12, 10 U/ml heparin; lanes 13−18, 100 mM Na_2SO_4. Panel **B**: The samples were immunoprecipitated with anti-diphtheria toxin (lanes 1−5) or anti-aFGF (lanes 6−20) and then analyzed by SDS−PAGE under reducing conditions. Before trypsin treatment the following compounds were added: lanes 1−10, no addition; lanes 11−16, 1 mM inositol hexaphosphate; lanes 17−20, 100 mM Na_2SO_4. Panel **C**: Aliquots (1 μl) of reticulocyte lysate containing [^{35}S]methionine-labelled dtA (lanes 1−13) or [^{35}S]methionine-labelled aFGF (lanes 14−26) were incubated in 10 μl HEPES medium, pH 4.5, with increasing concentrations of TPCK-treated trypsin as above and then analyzed by SDS−PAGE. Before trypsin treatment the following compounds were added: lanes 1−5 and 14−18, no addition; lanes 6−9 and 19−22, 1 mM inositol hexaphosphate; lanes 10−13 and 23−26, 100 mM Na_2SO_4.

in the present paper and as HBGF-1α (heparin-binding growth factor-1α) by Imamura *et al.* (1990) could be translocated when fused N-terminally to the A-fragment. The native structure of acidic fibroblast growth factor exhibits 12 antiparallel β strands arranged into a folding pattern with ~3-fold internal symmetry (Zhu *et al.*, 1991). While the protein as such assumes a relatively disordered (unfolded) form at physiological temperatures, it binds tightly to heparin and other polyanions, which induce a compact, folded conformation characterized by a dramatic increase in resistance to heat and proteases and in increased biological activity (Burgess and Maciag, 1989; Copeland *et al.*, 1991; Dabora *et al.*, 1991). We here demonstrate that a fusion protein of aFGF and diphtheria toxin A-fragment can be translocated to the cytosol and that heparin and other compounds that stabilize the growth factor prevent the translocation.

Results

Formation and characterization of the fusion protein

The gene encoding acidic fibroblast growth factor (aFGF) was fused to DNA encoding diphtheria toxin A-fragment and placed behind a T3 RNA polymerase promoter. The gene was transcribed and translated in a cell-free system in the presence of [^{35}S]methionine. On SDS−PAGE the fusion protein (aFGF-dtA) migrated corresponding to a molecular weight of 36 kDa as expected (Figure 1, lane 1). The protein was precipitated by anti-aFGF (lane 4) and by anti-diphtheria toxin (lane 5), but not by preimmune serum (lane 2) or by anti-ricin (lane 3).

When [^{35}S]methionine-labelled aFGF-dtA (lane 8) and [^{35}S]methionine-labelled diphtheria toxin B-fragment (dtB, lane 6) were mixed and dialyzed against PBS to remove reducing agents, the two proteins associated to form [aFGF-dtA-SS-dtB] (lane 9, arrow). Upon addition of reducing agent, the two proteins migrated separately again (lane 7), indicating that they were disulfide linked in the unreduced sample.

It is well established that aFGF binds to heparin and other polyanions and then assumes a more compact structure and acquires resistance to proteases (Burgess and Maciag, 1989; Copeland *et al.*, 1991; Sakaguchi *et al.*, 1991). Inorganic sulfate has also been shown to have such an effect (Dabora *et al.*, 1991). To test if this is also the case when the growth factor is linked to dtA, we dialyzed the ^{35}S-labelled aFGF-dtA with unlabelled dtB and tested the trypsin sensitivity of the [aFGF-dtA-SS-dtB] obtained in the absence and presence of heparin, inositol hexaphosphate or inorganic sulfate. The data in Figure 2A show that the labelled material in the untreated sample (lane 1) migrated in SDS−PAGE under reducing conditions at a rate corresponding to a mol. wt of ~36 kDa as expected for the fusion protein. At low concentrations of trypsin it was cleaved to yield a 24 kDa

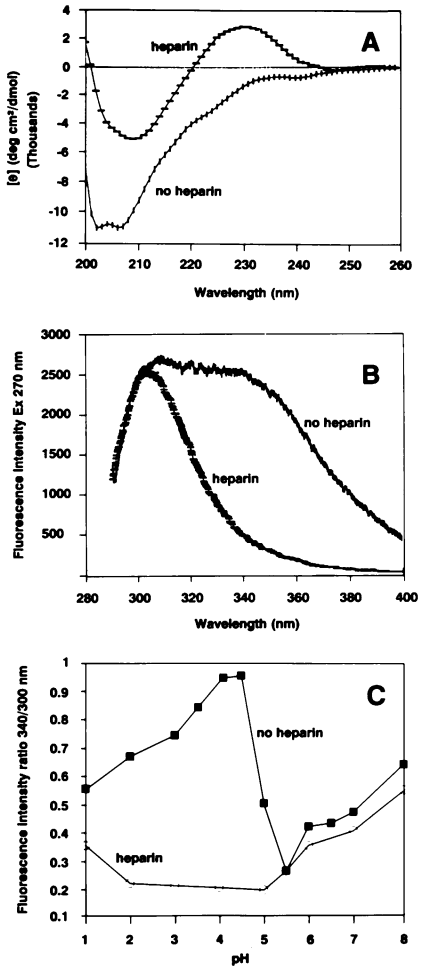

Fig. 3. Spectral analyses of aFGF in the absence and presence of heparin. (**A**) Far UV circular dichroism of human aFGF. Spectra were obtained at a protein concentration of 5 μM in 20 mM HEPES, 10 mM sodium acetate, pH 4.5, at 37°C, in the absence and presence of heparin at 1:1 molar ratio. (**B**) Fluorescence spectra of aFGF at pH 4.5, 37°C, in the absence and presence of heparin as in panel A. (**C**) Fluorescence intensity ratio at 340 and 300 nm of aFGF at various pH values in the absence and presence of heparin.

band, probably corresponding to the toxin A-fragment (21 kDa) and a small piece of the growth factor (there are several potential trypsin cleavage sites 14–30 amino acids from the C-terminus of the growth factor). This band was also obtained by immunoprecipitation with anti-dt, but not with anti-aFGF (Figure 2B). A band corresponding to the growth factor as such (mol. wt 15 kDa) was not obtained as expected, since there is no trypsin sensitive site between the growth factor and the A-fragment. However, at low trypsin concentration (panel A, lane 2) a band corresponding to 18 kDa was obtained, probably representing the growth factor and 24 amino acids of the A-fragment which contains a trypsin sensitive site at this position (Dumont and Richards, 1988). This band was immunoprecipitated by anti-aFGF, but not by anti-dt (panel B). At higher trypsin concentrations the growth factor was apparently degraded (panel A, lanes 3–6).

In the presence of heparin (panel A, lanes 7–12), inositol hexaphosphate (not demonstrated) or Na_2SO_4 (lanes 13–18), 10-fold higher trypsin concentrations were needed to cleave the fusion protein completely, and in this case an 18 kDa band was obtained even at high trypsin concentrations. It apparently represents the growth factor and the N-terminal part of the A-fragment. The rest of the A-fragment was degraded. The data indicate that heparin, inositol hexaphosphate and inorganic sulfate induce a more compact structure of aFGF in the fusion protein. The data in Figure 2C show that also aFGF alone, but not dtA did become resistant to trypsin in the presence of inositol hexaphosphate and sulfate.

Since toxin translocation occurs at low pH, the trypsin experiments demonstrated in Figure 2 were carried out at pH 4.5. However, heparin, inositol hexaphosphate and Na_2SO_4 also induced strong trypsin resistance of the aFGF part of the fusion protein at pH 7.0 (not demonstrated).

When heparin was added to the fusion protein before addition of B-fragment, the association with the B-fragment and formation of a disulfide bridge were not inhibited (data not shown), indicating that the growth factor folds like a separate domain, and does not alter the structure of the A-fragment to a great extent.

Effect of heparin on the structure of aFGF at various pH values

To study in more detail the effect of heparin on the structure of aFGF at various pH values, we carried out spectral analyses of the pure growth factor. As shown in Figure 3A, the far UV CD spectrum of aFGF at pH 4.5 manifests a strong negative ellipticity minimum at 203 nm, characteristic of disordered polypeptide chains. In contrast, in the presence of an equimolar concentration of heparin, this minimum is reduced in intensity and slightly shifted to a higher wavelength. In addition, a marked positive peak at 228 nm is now apparent. This latter spectrum of heparin-complexed aFGF is identical to that of the native folded protein (Copeland *et al.*, 1991).

Results from fluorescence experiments are consistent with these results (Figure 3B). The unfolded protein manifested strong tryptophan fluorescence near 340 nm at pH 4.5. When a 1:1 complex of aFGF was formed with heparin at this pH, the characteristic tyrosine fluorescence spectrum (emission maximum 303 nm) of the native protein was observed as formation of tertiary structure quenches the indole fluorescence (Copeland *et al.*, 1991).

We also examined the pH dependence of this phenomenon. Using the ratio of the fluorescence intensities at 340 and 300 nm as a measure of the degree of unfolding (Figure 3C) it can be seen that the ratio increases between pH 4.5 and 5.5, indicating that the protein undergoes a large conformational change between these two pH values in the absence of heparin. No such changes were observed in the presence of heparin. In summary, the data indicate that aFGF is extensively disordered at pH 4.5 and that heparin induces a more native-like structure at this pH. Similar experiments employing inositol hexaphosphate and inorganic phosphate as stabilizing polyanions produce similar results (not illustrated).

Binding to Vero cells of reconstituted toxin-containing fusion protein

The binding properties of the reconstituted protein [aFGF-dtA-SS-dtB] were measured on Vero cells, which are rich in diphtheria toxin receptors (Middlebrook *et al.*, 1978). As shown in Figure 4, [aFGF-dtA-SS-dtB] bound well in the absence (lane 1), but only weakly in the presence of excess

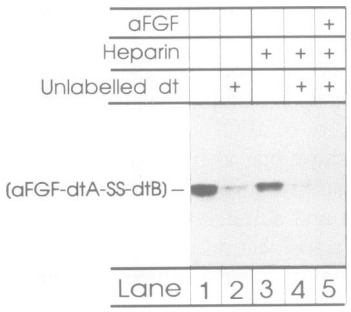

Fig. 4. Effect of heparin on the ability of [aFGF-dtA-SS-dtB] to bind to Vero cells. Reticulocyte lysate containing [^{35}S]methionine-labelled aFGF-dtA was mixed with lysate containing dtB and dialyzed to remove reducing agents. The disulfide-linked [aFGF-dtA-SS-dtB] was diluted 5-fold with HEPES medium and added in the absence and presence of the compound given below to Vero cells growing in 12-well microtiter plates and kept at room temperature for 20 min with gentle agitation. Then the cells were washed five times with HEPES medium and lysed with 300 μl of lysis buffer. The nuclei were removed by centrifugation and the TCA-precipitable material in the supernatant was analyzed by SDS–PAGE under nonreducing conditions and fluorography. The additions were: lane 1, none; lane 2, 10 μg/ml unlabelled diphtheria toxin; lane 3, 10 U/ml heparin; lane 4, 10 U/ml heparin and 10 μg/ml unlabelled diphtheria toxin; lane 5, 1 μg/ml unlabelled aFGF, 10 U/ml heparin and 10 μg/ml unlabelled diphtheria toxin.

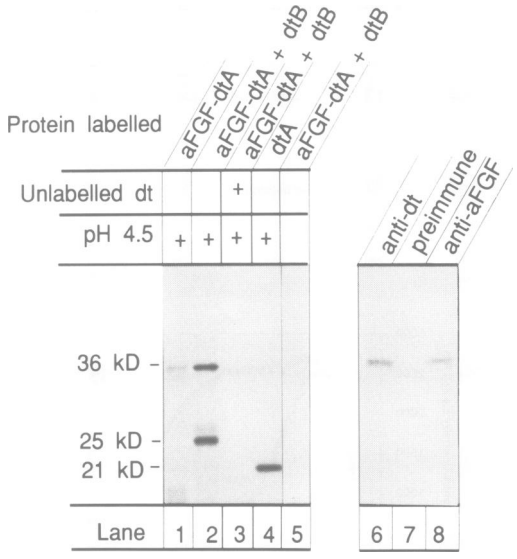

Fig. 5. Translocation of aFGF-dtA into cells. [^{35}S]methionine-labelled aFGF-dtA (lanes 1–3, 5–8) or [^{35}S]methionine-labelled dtA (lane 4) was mixed with unlabelled (lanes 1, 4 and 6–8) or [^{35}S]methionine-labelled dtB (lanes 2, 3 and 5) and dialyzed to form a disulfide-linked complex, which was then bound to Vero cells. Unlabelled diphtheria toxin (10 μg/ml) was present in one case (lane 3). Then the cells were exposed to pH 4.5 (lanes 1–4 and 6–8) or to pH 7.4 (lane 5) and subsequently treated with pronase to remove non-translocated material. The cells were washed and lysed and the nuclei were removed. In lanes 1–5 the TCA-precipitable material in the post-nuclear supernatant was collected, whereas in lanes 6–8 the supernatant was treated with protein A–Sepharose which had been pretreated with anti-diphtheria toxin (lane 6), preimmune serum (lane 7) or anti-aFGF (lane 8). In both cases the material was analyzed by SDS–PAGE under nonreducing conditions, followed by fluorography.

unlabelled diphtheria toxin (lane 2). In the presence of heparin alone the binding was somewhat reduced (lane 3), whereas the combination of heparin and unlabelled diphtheria toxin reduced it strongly (lane 4). The combination of heparin, unlabelled diphtheria toxin and aFGF completely abolished the binding (lane 5). aFGF alone did not visibly diminish the binding (data not shown), indicating that only a small proportion of the binding of the fusion protein was to aFGF receptors. The results indicate that [aFGF-dtA-SS-dtB] binds to Vero cells via the B-moiety to the diphtheria toxin receptor and via the aFGF part to surface heparans and to a small extent to specific fibroblast growth factor receptors.

Membrane translocation of the fusion protein

To study the translocation competence of [aFGF-dtA-SS-dtB], we bound the radiolabelled proteins to Vero cells and then exposed the cells briefly to acidic medium (pH 4.5). The cells were treated with pronase to remove non-translocated material and then subjected to SDS–PAGE to identify protected radiolabelled material.

As described earlier (Moskaug *et al.*, 1988), full-length diphtheria toxin gives rise to two protease protected polypeptides of 25 and 21 kDa, representing the C-terminal 230 residues of the B-fragment and the whole A-fragment, respectively (Moskaug *et al.*, 1988, 1991). Since the electrophoresis was carried out under nonreducing conditions, cell-mediated reduction of the interfragment disulfide bridge must have taken place. The reduction could conceivably occur upon exposure of the interfragment disulfide to the reducing cytosol (Moskaug *et al.*, 1987).

The data in Figure 5 show that only a 36 kDa polypeptide was obtained when only aFGF-dtA, but not dtB, was labelled (lane 1), whereas a 25 kDa polypeptide was also seen when dtB was labelled as well (lane 2). No protected fragments were obtained when specific binding to the diphtheria toxin receptors was inhibited by the presence of excess unlabelled toxin (lane 3) or when the treatment with low pH was omitted (lane 5). When the translocation experiment was carried out with reconstituted diphtheria toxin alone where only the A-fragment was labelled, only a 21 kDa protected band was seen (lane 4), which represents the translocated A-fragment (Stenmark *et al.*, 1992).

It has been reported that aFGF may bind strongly to a 24 kDa cellular protein to form an SDS resistant complex of 40 kDa (Shi *et al.*, 1991). To test if the protected 36 kDa material shown in lanes 1 and 2 consisted of both aFGF and dtA we carried out immunoprecipitation experiments. The pronase protected material was precipitated by anti-aFGF (lane 8), but not by serum from a non-immunized rabbit (lane 7). Most importantly, it was also precipitated by anti-diphtheria toxin (lane 6), excluding the possibility that the protected material was a complex of aFGF and a cellular protein. Altogether, the data indicate that aFGF-dtA is translocated into the cells when associated with B-fragment, bound to cells and then exposed to low pH.

The possibility existed that the protected aFGF-dtA was stuck in the membrane rather than free in the cytosol. To study this, we treated the cells with low concentrations of saponin to allow cytoplasmic proteins to diffuse out of the cells and into the medium. After centrifugation the released proteins are present in the supernatant, whereas membrane associated proteins are obtained in the pellet (Moskaug *et al.*, 1988). In the results shown in Figure 6, all of the 25 kDa polypeptide and part of aFGF-dtA (36 kDa) was obtained in the pellet (lane 3) whereas approximately half of the aFGF-dtA, but no 25 kDa polypeptide, was found in the

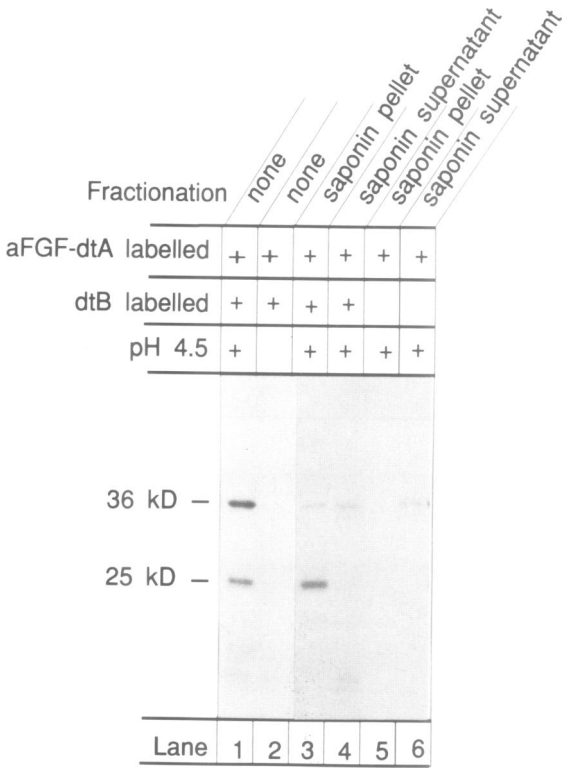

Fig. 6. Saponin fractionation of cells with translocated aFGF-dtA. [aFGF-dtA-SS-dtB] labelled in one or both moieties as indicated was bound to Vero cells. Then the cells were exposed to pH 4.5 (lanes 1 and 3–6) or to pH 7.4 (lane 2) and then treated with pronase. After washing, the cells were treated with 50 μg/ml saponin (to release proteins that were free in the cytosol) and centrifuged. The membrane pellet and the TCA-precipitable material in the supernatant were analyzed by nonreducing SDS–PAGE, and fluorography.

supernatant (lane 4). This amount varied somewhat between experiments; in the experiment shown in lanes 5 and 6, aFGF-dtA was detected only in the supernatant (lane 6) and not in the pellet (lane 5). In this experiment only aFGF-dtA was labelled and then associated with unlabelled dtB, which is the reason why no labelled 25 kDa fragment was obtained in lane 5. Altogether, it appears that most of the aFGF-dtA diffused out of the cells upon saponin treatment in the same way as natural A-fragment (Moskaug *et al.*, 1988), whereas most of the dtB-derived 25 kDa polypeptide was retained in the membranes. The data indicate that most aFGF-dtA is translocated into the cytosol, without being arrested in the membrane.

Effect of heparin, inositol hexaphosphate and inorganic sulfate on translocation of the fusion protein

Since aFGF linked to dtA appeared to assume a more compact structure in the presence of heparin than in its absence (Figure 2), we tested the possibility that heparin could interfere with the translocation process. This was indeed found to be the case. When [aFGF-dtA-SS-dtB] was labelled with [^{35}S]methionine in both moieties, both a 36 kDa and a 25 kDa polypeptide were protected in the absence of heparin (Figure 7, lane 1), whereas only the 25 kDa polypeptide was protected when heparin was present in the medium (lane 2). When only the aFGF-dtA moiety was labelled, no protected polypeptide was seen in the presence of heparin (lane 3).

In the next series of experiments we carried out

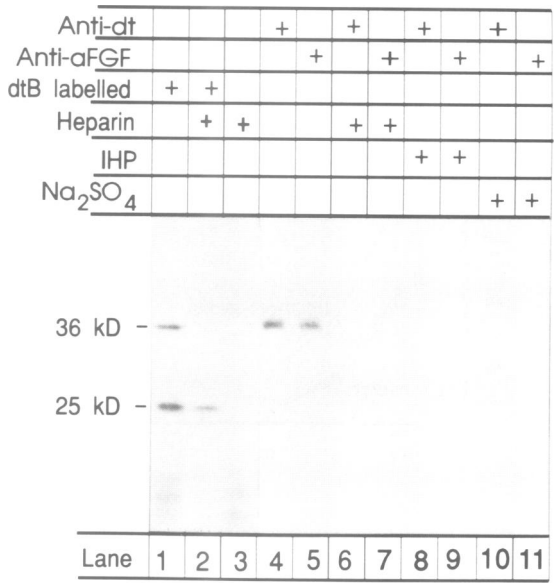

Fig. 7. Ability of heparin, inositol hexaphosphate and Na_2SO_4 to prevent translocation of aFGF-dtA to the cytosol. [aFGF-dtA-SS-dtB] labelled with [^{35}S]methionine in both moieties (lanes 1 and 2) or only in aFGF-dtA (lanes 3–11) was bound to cells for 2 h at 0°C and then 10 U/ml heparin or 1 mM inositol hexaphosphate was added, or the cells were transferred to medium in which 100 mM NaCl had been replaced by 100 mM Na_2SO_4. After 15 min, the cells were exposed to pH 4.5 in the absence or presence of the same compounds. Then the cells were treated with pronase, washed and lysed and the post-nuclear supernatant was collected. In lanes 4–11 is shown the material adsorbed to protein A–Sepharose pretreated with anti-aFGF (lanes 1, 5, 7, 9 and 11) or anti-diphtheria toxin (lanes 4, 6, 8 and 10) and analyzed by nonreducing SDS–PAGE and fluorography.

immunoprecipitations of the lysates obtained from cells after pronase treatment (lanes 4–11). Only the aFGF-dtA moiety was labelled in these experiments. With both anti-aFGF (lane 5) and anti-diphtheria toxin (lane 6) a 36 kDa polypeptide was precipitated when the translocation had been induced in the absence of heparin, whereas when heparin was added before exposure to low pH no radioactive material was precipitated from the cell lysate with anti-aFGF (lane 7) or with anti-diphtheria toxin (lane 6). Altogether, the data indicate that binding of heparin to aFGF-dtA completely blocked translocation via the diphtheria toxin translocation apparatus, although the insertion of the B-fragment was not inhibited.

Heparin is a heterogenous mixture of sulfated glucosaminoglycans (mol. wt 5000–30 000) consisting of repeating disaccharide subunits of hexuronic acid and D-glucosamine (Lindahl, 1989). It was therefore possible that the tight binding to these molecules could block the translocation mechanically. We therefore repeated the experiment with the much smaller inositol hexaphosphate (mol. wt 660) which would be expected to offer less mechanical hindrance. As shown in Figure 7, no protected material could be immunoprecipitated from the cell lysate with anti-aFGF (lane 9) or with anti-diphtheria toxin (lane 8), indicating that inositol hexaphosphate blocked the translocation in the same way as heparin. The same results were obtained in the presence of 100 mM Na_2SO_4 (lanes 10 and 11).

Control experiments showed that heparin, inositol hexaphosphate and Na_2SO_4 in the concentrations here used did not inhibit translocation of diphtheria toxin as such (data not shown).

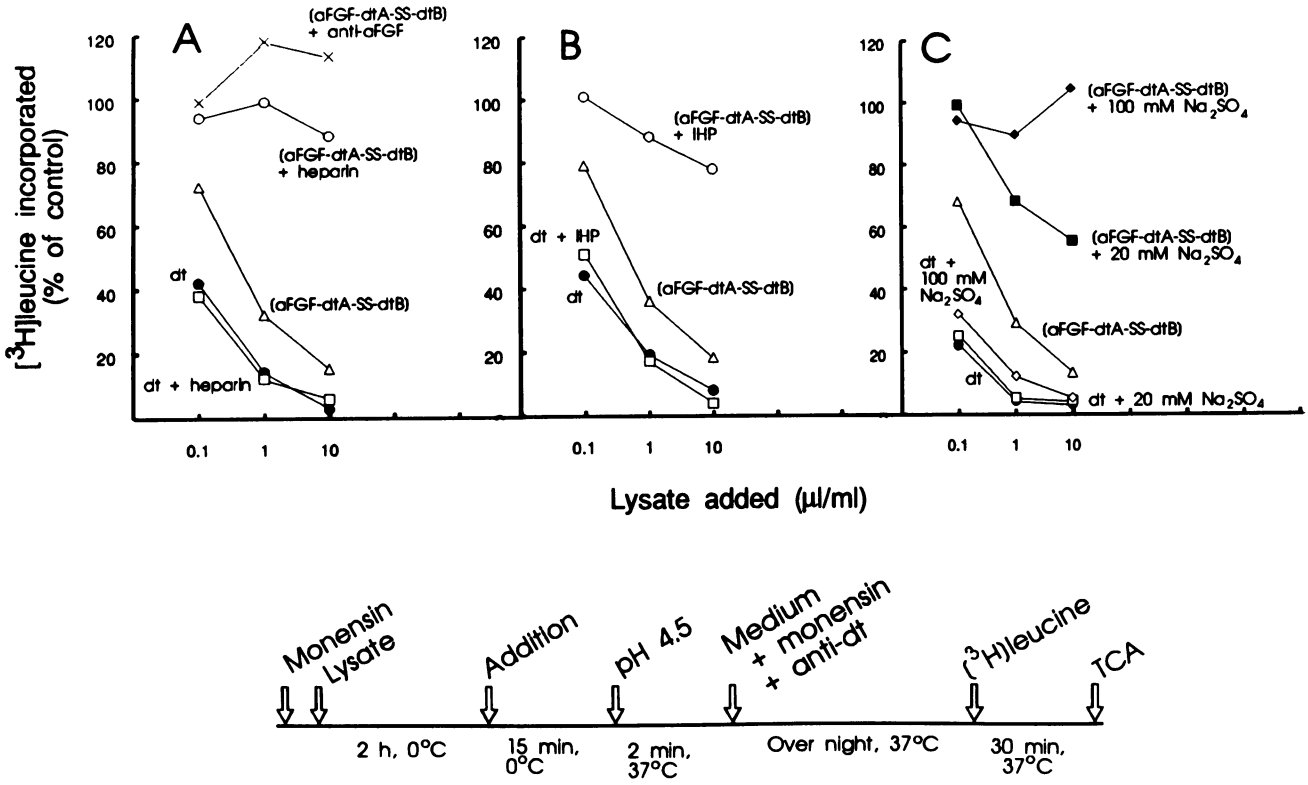

Fig. 8. Effect of heparin, inositol hexaphosphate and Na_2SO_4 on the cytoxicity of [aFGF-dtA-SS-dtB]. To Vero cells in HEPES medium containing 10 µM monensin, increasing amounts of rabbit reticulocyte lysate containing 500 ng/ml unlabelled reconstituted diphtheria toxin or [aFGF-dtA-SS-dtB] was added. After 2 h at 0°C the lysates were removed and HEPES medium containing heparin, inositol hexaphosphate and Na_2SO_4 as indicated and 10 µM monensin were added. After 15 min at 0°C, the medium was removed and the cells were exposed to HEPES medium, pH 4.5, containing heparin, inositol hexaphosphate and Na_2SO_4 as above, and 10 mM sodium gluconate but no monensin, for 2 min at 37°C. Then growth medium containing 10 µM monensin and 1 µl/ml horse anti-diphtheria toxin was added and the cells were incubated overnight at 37°C. Finally, the ability of the cells to incorporate [^{3}H]leucine during 30 min was measured. **A**: ●, reconstituted diphtheria toxin (from *in vitro*-made dtA and dtB); □, reconstituted diphtheria toxin and 10 U/ml heparin; △, [aFGF-dtA-SS-dtB]; ○, [aFGF-dtA-SS-dtB] and 10 U/ml heparin; x, [aFGF-dtA-SS-dtB] and 2 µg/ml anti-aFGF. **B**: ●, reconstituted diphtheria toxin; □, reconstituted diphtheria toxin and 1 mM inositol hexaphosphate; △, [aFGF-dtA-SS-dtB]; ○, [aFGF-dtA-SS-dtB] and 1 mM inositol hexaphosphate. **C**: ●, reconstituted diphtheria toxin; □, reconstituted diphtheria toxin and 20 mM Na_2SO_4; ◇, reconstituted diphtheria toxin and 100 mM Na_2SO_4; △, [aFGF-dtA-SS-dtB]; ■, [aFGF-dtA-SS-dtB] and 20 mM Na_2SO_4; ◆, [aFGF-dtA-SS-dtB] and 100 mM Na_2SO_4.

Toxicity of diphtheria toxin fused with aFGF

Diphtheria toxin reconstituted from fragments A and B made in a reticulocyte lysate system has been shown to be fully toxic to Vero cells (Stenmark *et al.*, 1992). This is also evident from Figure 8. The presence of heparin in the medium did not diminish the toxic effect (Figure 8A). [aFGF-dtA-SS-dtB] was also toxic, although somewhat less so than unconjugated toxin. This supports the findings above that aFGF-dtA is translocated to the cytosol. The addition of anti-aFGF before exposure to low pH prevented the toxic effect, excluding the possibility that the toxicity was due to molecules in which aFGF had been split off from dtA by proteolysis before the translocation. When heparin was present during the exposure to low pH, the cells were protected against [aFGF-dtA-SS-dtB], supporting the conclusion drawn from the data above that heparin inhibits translocation.

The data in Figure 7B show that also 1 mM inositol hexaphosphate protected the cells efficiently against the fusion protein, but not against diphtheria toxin. Furthermore, 100 mM Na_2SO_4 protected cells strongly against intoxication by the fusion protein, whereas 20 mM Na_2SO_4 protected to a lesser extent (Figure 8C). Altogether, the data indicate that compounds that stabilize aFGF by inducing a more tight folding prevent its translocation to the cytosol along with diphtheria toxin.

Discussion

The main finding presented here is that aFGF fused to diphtheria toxin can be translocated to the cytosol along with the toxin A-fragment in the absence, but not in the presence of heparin, inositol hexaphosphate and inorganic sulfate. It is well established that heparin and other polyanions protect aFGF against a number of proteases and against denaturation by pH and by heat (Burgess and Maciag, 1989; Dabora *et al.*, 1991). The protective effect is most apparent above 30°C and it has been suggested that polyanions stabilize the native conformation of aFGF at physiological temperatures where it otherwise assumes a more disordered structure. We show here that techniques sensitive to secondary and tertiary structure (circular dichroism and fluorescence respectively) indicate that aFGF is relatively unfolded at pH 4.5 in the absence of polyanions, but assumes a compact, native conformation in the presence of heparin.

The requirement for aFGF to be in a non-native, disordered conformation in order to be translocated across the membrane along with diphtheria toxin A-fragment

resembles the requirement for translocation of mitochondrial precursor proteins, eukaryotic pre-secretory proteins and bacterial precursor proteins (Sanz and Meyer, 1988; Verner and Lemire, 1989; Watanabe and Blobel, 1989; Rassow *et al.*, 1990; Pfanner *et al.*, 1990; Skerjanc *et al.*, 1990). aFGF has a three-dimensional structure similar to that of basic FGF (Zhu *et al.*, 1991) and to that of interleukin 1α and β (Eriksson *et al.*, 1991; Zhang *et al.*, 1991). All these proteins are synthesized as cytosolic proteins without a signal sequence (Jaye *et al.*, 1986; Muesch *et al.*, 1990) despite their extracellular site of action which consists in binding to specific cell surface receptors (Burgess and Maciag, 1989). It is not known how they are transported out of the cells (Muthukrisnan *et al.*, 1991; Mignatti *et al.*, 1991), but their ability to assume a less ordered conformation at physiological temperatures could be important for this process.

It should be noted that aFGF contains three cysteine residues in a reduced state which are apparently not required for biological activity (Ortega *et al.*, 1991). In the reconstitution experiments with the B-fragment these SH-groups are presumably not involved. Thus, we have shown (Stenmark *et al.*, 1992) that A-fragment extended with various peptides and proteins lacking cysteine efficiently associate with B-fragment. Furthermore, the presence of heparin did not inhibit reassociation. Finally, dialysis of aFGF alone did not result in oligomer formation, as would be expected if the free SH-groups were easily accessible for disulfide formation.

We therefore feel that the construct used in the present experiments was composed of B-fragment of diphtheria toxin reassociated with the A-fragment part of the fusion protein. It is likely that both moieties of the fusion protein retained their normal properties. Thus, when translocated to the cytosol, the A-fragment part inhibited protein synthesis, and the fusion protein could be immunoprecipitated both with anti-aFGF antibody and with anti-diphtheria toxin serum.

The binding experiments also indicate that the different parts of [aFGF-dtA-SS-dtB] retained their normal activities. Thus, the construct was bound via the B-fragment to diphtheria toxin receptors at the cell surface, and via the growth factor both to the extracellular heparin-like structures and to specific aFGF receptors. Although the binding of [aFGF-dtA-SS-dtB] to cells was somewhat reduced in the presence of heparin, this cannot account for the complete prevention of translocation of the fusion protein in the presence of heparin and other polyanions, measured both as translocated radioactive protein and as inhibition of protein synthesis. Furthermore, the finding that no radioactive material was translocated in the presence of excess unlabelled toxin indicates that only molecules bound to the diphtheria toxin receptor are translocated to the cytosol whereas the rest of the cell surface bound molecules were degraded during the pronase treatment.

In the toxicity experiments, [aFGF-dtA-SS-dtB] was somewhat less efficient in inhibiting protein synthesis in the cells than toxin without the growth factor. Since both binding to the diphtheria toxin receptors and the extent of translocation to the cytosol appeared to be the same in the two cases, the reason is probably that the enzymatic activity of dtA is somewhat reduced when linked to the growth factor. Alternatively, the growth factor could be removed proteolytically from part of the fusion protein molecules after translocation and only then aquire enzymatic activity. We consider this less likely, as a 4 h incubation of the cells after translocation did not result in disappearance of the fusion protein (unpublished data).

From the present data indicating that aFGF must be in a relatively unfolded state to be translocation competent, it does not necessarily follow that dtA unfolds during translocation. The observation that the A-fragment assumes a more loosely folded structure at the low pH required for translocation (Dumont and Richards, 1988) suggests, however, that this is indeed the case. Possibly, once the translocation process is initiated, any protein capable of unfolding may be translocated along with the A-fragment.

Materials and methods

Materials, media and buffers

Acidic FGF was obtained from Boehringer Mannheim Biochemie, Germany. HEPES medium: bicarbonate-free Eagle's minimum essential medium buffered with 20 mM HEPES (*N*-2- hydroxethylpiperazine-*N'*-2-ethanesulfonic acid) and adjusted to the pH indicated. When the pH was adjusted to below 5.3, 10 mM sodium gluconate was added to increase the buffering capacity. PBS: 140 mM NaCl, 10 mM Na_2HPO_4, pH 7.2. Lysis buffer: PBS containing 10 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.1 mM sodium vanadate, 200 U/ml aprotinin, 1 mM PMSF, 1 mM NEM. Recombinant human aFGF was obtained and characterized as described by Copeland *et al.* (1991).

Plasmid construction

Escherichia coli strains DH5α and JM105 were used in the cloning procedures. To form pHBFG-dt1, an *Nco*I site was generated in each end of the HBFG-1α gene (Jaye *et al.*, 1986; Imamura *et al.*, 1990) by PCR using *Taq*I DNA polymerase with the primer G GCC ATG GCT AAT TAC AAG AAG CCC AAA in the forward direction and with GGC CAT GGA ATC AGA AGA GAC TGG CAG GGG as primer in the reverse direction. The PCR product was gel isolated, partially cut with *Nco*I (the HBGF-1α gene has an internal *Nco*I site), gel isolated again and then cloned into pBD-30 which had been linearized with *Nco*I and then dephosphorylated. pBD-30 encodes the A-fragment of diphtheria toxin behind a T3 RNA polymerase promoter. pBD-23 encodes the B-fragment of diphtheria toxin behind a T3 promoter (Stenmark *et al.*, 1992).

Cell culture

Vero cells were maintained and propagated under standard conditions (5% CO_2 in Eagle's minimal essential medium containing 5% FCS). Two days prior to the experiments, the cells were seeded into 12- or 24-well Costar plates at a density of 5×10^4 and 10^5 cells/well, respectively.

SDS – PAGE

SDS–PAGE was carried out in 12% gels as described by Laemmli (1970). The gels were fixed in 4% acetic acid/27% methanol for 30 min and then treated with 1 M sodium salicylate, pH 5.8, in 2% glycerol for 30 min, to visualize proteins labelled with [^{35}S]methionine. Dried gels were exposed to Kodak XAR-5 films in the absence of intensifying screens at −80°C for fluorography.

Spectral analyses

CD spectra were obtained with a Jasco J720 spectropolarimeter. Samples of recombinant aFGF (5 μM in 20 mM HEPES buffer, pH adjusted with acetic acid as indicated) were examined in 0.1 cm pathlength cells with the temperature of the cell holder controlled by a circulating water bath. Fluorescence spectra were measured with a Hitachi F4500 fluorimeter with 270 nm excitation at 5 nm resolution and the temperature was controlled as above. All spectra were corrected for background fluorescence and Raman scattering. Identical samples were used in the CD and fluorescence studies. Fluorescence spectra as a function of pH were obtained in a buffer containing 50 mM glycine, 50 mM sodium acetate and 50 mM sodium phosphate, and the pH was adjusted with concentrated HCl and NaOH. Further discussion of these methods and their specific application to aFGF are presented in Copeland *et al.* (1991).

In vitro transcription and translation

The plasmids were linearized downstream of the inserts by *Eco*RI, and transcribed *in vitro* with T3 polymerase (McGill *et al.*, 1989). The transcripts

were translated for 1 h at 30°C in a rabbit reticulocyte lysate system (Promega, WI, USA) in the presence of 0.25 mCi (1 μM) of [^{35}S]methionine (1000 Ci/mmol, Amersham). In some cases 25 μM unlabelled methionine was used instead of [^{35}S]methionine. The concentrations of the translation products obtained were estimated as described by Stenmark *et al.* (1992). After translation, the lysates were dialyzed at 4°C first for 16 h against PBS and then for 4 h against HEPES medium, to remove free [^{35}S]methionine and the reducing agent, allowing disulfide bridges to be formed. The lysates were then analyzed by SDS–PAGE (under reducing and non-reducing conditions as indicated) and fluorography. Translation mixtures were either used undiluted or diluted with HEPES medium, pH 7.2.

Cell binding and translocation assay

To measure binding, dialyzed translation mixture (diluted 5 times in HEPES medium) was added to Vero cells growing as monolayers in 12-well microtiter plates and kept at 24°C for 20 min in the presence of 1 mM unlabelled methionine and 10 μM monensin (Moskaug *et al.*, 1988). To measure translocation, undiluted, dialyzed lysates were added to cells and kept for 20 min at 24°C in the presence of 1 mM methionine and 10 μM monensin. The cells were then washed three times with ice-cold HEPES medium and exposed to pH 4.5, treated with pronase and analyzed by SDS–PAGE and fluorography as described (Moskaug *et al.*, 1988). In some cases the cells were treated with 50 μg/ml saponin at 4°C for 30 min to separate the membrane fraction from the cytosol as described (Moskaug *et al.*, 1988).

Immunoprecipitation

Aliquots of 25 μl protein A–Sepharose (Pharmacia, Uppsala, Sweden) were incubated with 2 μl of rabbit anti-diphtheria toxin serum or rabbit anti-bovine aFGF (Promega, Madison, WI) for 30 min at ambient temperature and then washed twice with lysis buffer. Samples (200 μl) of cell lysate or diluted translation products were then added and kept at 4°C for 60 min with rotation. The pellets were subsequently washed three times with lysis buffer and once with water, and finally subjected to SDS–PAGE.

Measurements of protein synthesis inhibition

Increasing amounts of diphtheria toxin or fusion protein were added to cells and translocation was then induced by exposure to pH 4.5 as described above. Medium containing monensin (to prevent translocation from endosomes) and 1 μl/ml of anti-diphtheria toxin antiserum was then added and the cells were incubated overnight. Finally, the rate of protein synthesis was measured as the amount of [^{3}H]leucine incorporated into acid-insoluble material (Sandvig and Olsnes, 1982).

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