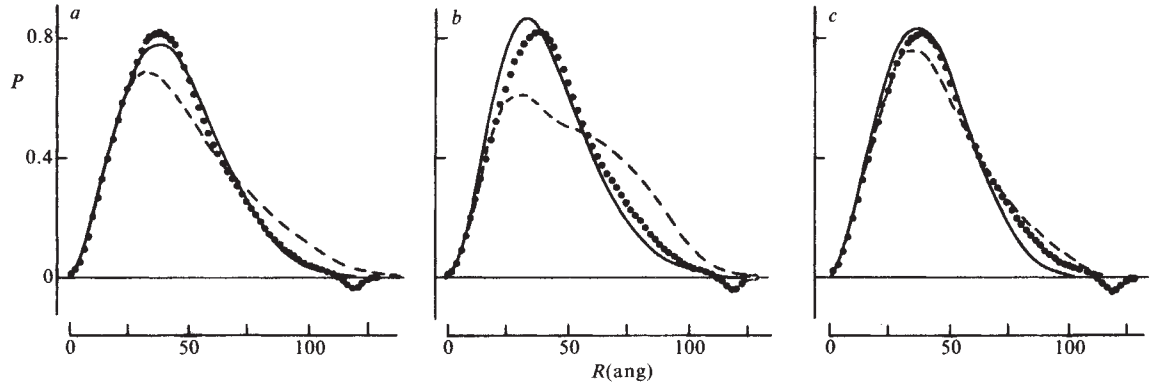

Fig. 3 A comparison of the S-1 experimental (●) chord distribution with the predictions from electron microscopy using the Taylor and Amos interpretation (solid lines) and the Moore *et al.* interpretation (dashed lines). The electron density maps were from: *a*, Moore *et al.*; *b*, Taylor and Amos; *c*, Wakabayashi and Toyoshima. The maps were furnished with the actin–S-1 interfaces delimited by their authors except in the case of the Moore *et al.* map, where the interface was delimited by L. Amos. The maps were scaled to the correct volume. This was done by scaling each x and y coordinate or each x, y and z (helix axis) coordinate as recommended by the author. Typically, the scaling changed these dimensions by only 10–20%. Tests showed that the method of scaling had only a small effect on the predicted chord distributions and on the overall molecular shape (see Table 1). Chord distributions were obtained by Fourier transforming the experimental and model scattering curves using the method of Glatter[14].

S-1 solution could have a shape similar to that found by Taylor and Amos and yet makes a conformational change to the shape described by Moore on binding to actin[7], such a large change seems improbable because the accompanying R_g change would be of an unprecendented magnitude. Thus, the present results are consistent with the notion that regulation of muscle contraction occurs by the steric blocking of the actin–S-1 interaction.

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1. Haselgrove, J. C. *Cold Spring Harb. Symp. quant. Biol.* **37**, 341–352 (1972).
2. Huxley, H. E. *Cold Spring Harb. Symp. quant. Biol.* **37**, 361–376 (1972).
3. Parry, D. A. D. & Squire, J. *J. molec. Biol.* **75**, 33–55 (1973).
4. Seymour, J. & O'Brien, E. J. *Nature* **283**, 680–682 (1980).
5. Moore, P. B., Huxley, H. E. & De Roosier, D. J. *J. molec. Biol.* **50**, 279–295 (1970).
6. Taylor, K. A. & Amos, L. *J. molec. Biol.* **147**, 297–324 (1981).

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7. Wakabayashi, T. & Toyoshima, C. *J. Biochem.* **90**, 683–701 (1981).
8. Mendelson, R. A. & Kretzschmar, K. M. *Biochemistry* **19**, 4103–4108 (1980).
9. Vibert, P. & Craig, R. *J. molec. Biol.* **157**, 299–319 (1982).
10. Margossian, S. S., Lowey, S. & Barshop, B. *Nature* **258**, 163–165 (1975).
11. Weeds, A. G. & Taylor, R. S. *Nature* **257**, 54–56 (1975).
12. Margossian, S. S., Stafford, W. III & Lowey, S. *Biochemistry* **20**, 2151–2155 (1981).
13. Craig, R. *et al. J. molec. Biol.* **140**, 35–55 (1980).
14. Glatter, O. *J. appl. Crystallogr.* **10**, 415–421 (1977).

Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system

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Insulin forms a complex with its receptors on the plasma membrane of cells and initiates a series of biochemical events which lead to the hormone's recognized effects[1,2]. The exact mechanism that initiates these events is unknown. We previously reported that insulin stimulates the phosphorylation of the 95,000 molecular weight (M_r) (β) subunit of its own receptor in intact cells and proposed that this phosphorylation reaction could be a very early step in insulin action[3]. To clarify the molecular basis of this reaction, we have now investigated the phosphorylation of insulin receptor in a cell-free system. Using [γ-^{32}P]ATP in solubilized and partially purified receptor preparations from rat liver plasma membrane, we find that both the α and β subunits of the insulin receptor are phosphorylated. Furthermore, insulin stimulates the incorporation of ^{32}P into both receptor subunits in a specific and dose-dependent manner. Phosphoamino acid determination of the β subunit after insulin stimulation reveals only phosphotyrosine. These findings suggest that the elements required for phosphorylation are associated with the plasma membrane of the cell and that specific phosphorylation of the insulin receptor on tyrosine residues can be activated in a solubilized preparation.

Rat liver plasma membranes were prepared by the procedure of Neville[4] and phosphorylation studied using [γ-^{32}P]ATP as described in Fig. 1 legend. Insulin receptors in this rat liver membrane fraction and solubilized insulin receptors prepared from these membranes have been well characterized[5,6]. Further, as previously shown lectin chromatography allows a 20-fold purification with nearly 100% recovery of insulin receptors as determined by ^{125}I-insulin binding[7]. The insulin receptor subunits were identified by immunoprecipitation with antibodies against insulin receptor followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography[3,8].

When the immunoprecipitates were analysed in SDS-PAGE in reducing conditions (100 mM dithiothreitol; DTT), one major band of M_r 95,000 and a minor band of M_r 135,000 were specifically phosphorylated in basal state (Fig. 1*A*, lane *b*). These 135,000- and 95,000-M_r phosphoproteins have been tentatively identified as the α and β subunits of insulin receptor, respectively, by their immunoprecipitation by anti-receptor antibody and by the fact that they migrate in the same position in SDS-PAGE as α and β subunits of insulin receptor labelled either biosynthetically or externally[3,8]. After incubation of the solubilized fraction with insulin (10^{-6} M), the incorporation of ^{32}P into these two proteins was increased approximately fivefold as determined by scanning densitometry (Fig. 1*A*, lane *d*). A phosphoprotein band corresponding to $M_r \sim 50{,}000$ is also observed; this may represent phosphorylation of the heavy chain of IgG.

In the native insulin receptor, the receptor subunits are thought to be linked by disulphide bonds to form high molecular weight oligomers[9,10]. When the same samples were analysed without reduction of disulphide bonds, five bands were observed (Fig. 1*B*, lane *b*). Two bands had molecular weights higher than 300,000. In addition, there were phosphoproteins of M_rs 240,000, 130,000 and 90,000 in basal state (Fig. 1*B*, lane *b*); these bands are identical to those found when the receptor

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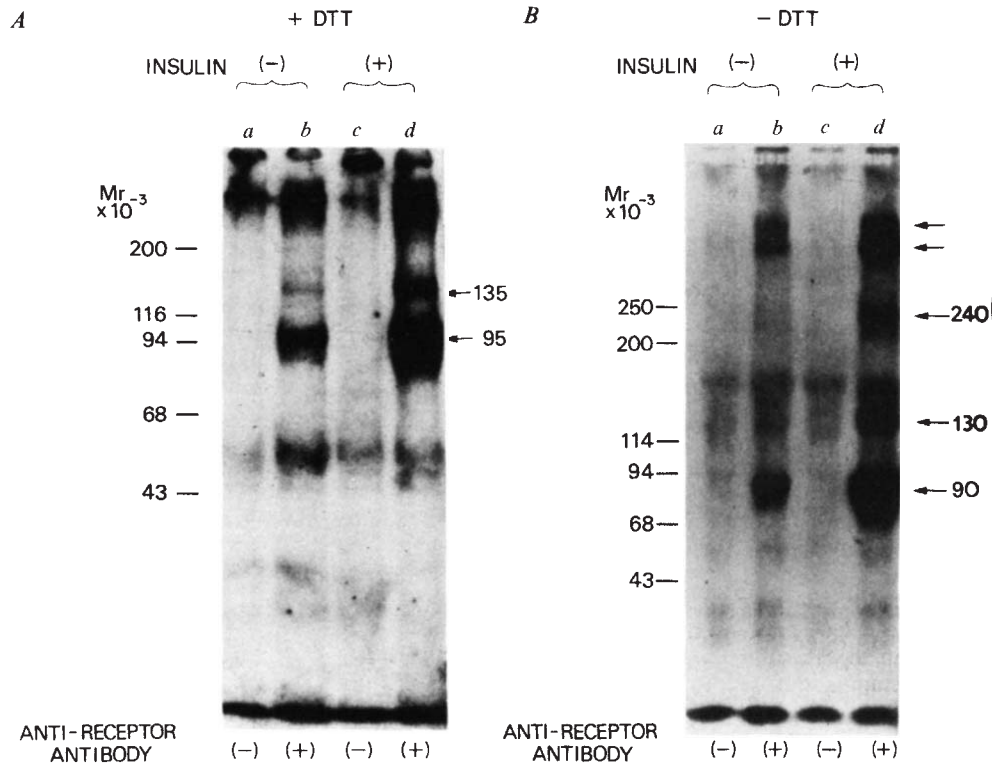

Fig. 1 Autoradiogram showing the incorporation of the ^{32}P from [γ-^{32}P]ATP into solubilized insulin receptor from rat liver plasma membrane. Rat liver plasma membranes were prepared by the procedure of Neville[4]. Protease inhibitors (2 mM phenylmethylsulphonyl fluoride; 1,000 trypsin inhibitor units per ml aprotinin) were added throughout the purification beginning with the first step of homogenization. The plasma membrane fraction was solubilized by 1% (v/v) Triton X-100 in 50 mM HEPES buffer, *p*H 7.6, containing protease inhibitors. The insulin receptors were further enriched by chromatography on a wheat germ agglutinin-agarose column[7] after elution by 0.3 N *M*-acetyl glucosamine in 50 mM HEPES buffer, *p*H 7.6, containing 0.1% (v/v) Triton X-100. Aliquots of these eluted fractions (150–300 μg per tube) were incubated with or without insulin (10^{-6} M) overnight at 4 °C. A 1-h incubation with insulin at room temperature was also used. Essentially the same result was obtained in the two different incubation conditions. The aliquots were then assayed for the incorporation of ^{32}P from [γ-^{32}P]ATP at 4 °C in a final volume of 1,350 μl. For the experiment shown, the reaction mixture contained 6 mM $MgCl_2$, 2 mM $Mn(CH_3COO)_2$, 5 μM [γ-^{32}P]ATP (~30 μCi nmol^{-1}) in 30 mM HEPES buffer, *p*H 7.6. Similar results were obtained with 20 mM $MgCl_2$ and 1 mM $MnCl_2$ in a 100 mM HEPES buffer, *p*H 7.4. Assays were initiated by the addition of ATP. After 10 min incubation, NaF, sodium pyrophosphate, EDTA, ATP and Triton were added to get final concentration of 50, 10, 5 and 5 mM and 0.1% (v/v) respectively. The insulin receptor was quantitatively immunoprecipitated by serum containing antibody against insulin receptor (serum B-2, 1:200 dilution) or normal control serum[8]. After 2.5 h at 4 °C, 200 μl of Protein A (Pansorbin; 10% w/v) were added and the incubation continued for 1 h at 4 °C. The precipitates were then collected by centrifugation at 10,000*g* for 5 min at 4 °C and washed twice with 1% Triton and 0.1% SDS[8]. Immunoprecipitates were solubilized by boiling for 3 min in 2% SDS in 10 mM sodium phosphate, *p*H 7.0, with (*A*) or without (*B*) dithiothreitol (DTT). The labelled components were separated electrophoretically in 5 or 7.5% polyacrylamide slab gels in the presence of 0.1% SDS as described by Laemmli[32]. After electrophoresis, the slab gels were stained, destained, dried and autoradiographed for 3–7 days as previously described[8]. Subunit molecular weights were calculated by using as standards (M_r): filamin (250,000), myosin (200,000), β-galactosidase (116,000), phosphorylase *b* (94,000), bovine serum albumin (68,000) and ovalbumin (43,000).

is labelled by ^{35}S-methionine or ^{3}H-sugars and analysed without reduction[11]. Using two-dimensional (non-reducing/reducing) gel electrophoresis, we have previously shown that the two highest molecular weight bands are composed of α and β subunits, and three other bands are an α–α dimer and 'free' α and β subunits respectively[11]. After incubating with insulin (10^{-6} M), the phosphorylation of all five bands was increased approximately five- to six-fold.

To evaluate further the mechanism of phosphorylation of insulin receptors, the solubilized fractions were incubated with several concentrations of insulin (10^{-10} to ~10^{-6} M), desoctapeptide (DOP) insulin and multiplication-stimulating activity (MSA), one of the insulin-like growth factors, and the phosphorylation was carried out as described above. Physiological concentrations of insulin (3×10^{-10} M) increased the phosphorylation of both subunits approximately threefold (Table 1). Stimulation was maximal with insulin concentrations of 10^{-8} to ~10^{-6} M (Table 1; Fig. 1). DOP insulin, which has ≤1% of the potency of porcine insulin in displacing bound ^{125}I-insulin from rat liver membrane insulin receptor and stimulating glucose metabolism[6], stimulated phosphorylation only minimally. MSA (10^{-7} M) was also far less potent than the same concentration of porcine insulin (Table 1), as expected from previous studies of the affinity of MSA for the insulin receptor in rat liver plasma membrane[12,13]. Thus, the 135,000- and 95,000-M_r bands were phosphorylated in dose-dependent fashion and specificity consistent with the affinity of the insulin receptor for insulin analogues.

To determine the phosphoamino acids of the β subunit of the insulin receptor, this ^{32}P-labelled phosphoprotein was cut from the gel and partially hydrolysed in 6 M HCl for 2 h at 110 °C. The resulting hydrolysates were separated by two-dimensional electrophoresis (*p*H 2.0 and *p*H 3.5) in the presence of unlabelled phosphoserine, phosphothreonine and phosphotyrosine, and the autoradiograms of the separated phosphoamino acids were compared with the ninhydrin-stained standards. Only trace amounts of phosphotyrosine could be detected in the basal state (data not shown). After insulin stimulation (10^{-7} M), there was a dramatic increase in the amount of phosphotyrosine (Fig. 2). Because of the low radioactivity, phosphoamino acid in α subunit could not be identified.

There is considerable evidence that phosphorylation and dephosphorylation may have various roles in insulin action[14–20]. Recently, we found that insulin stimulates the phosphorylation

Table 1 Effects of insulin and insulin analogues on ^{32}P incorporation into solubilized insulin receptor subunits from rat liver plasma membrane

Addition	Concentration (M)	^{32}P incorporated into insulin receptor subunits (arbitrary units)	
		M_r 135,000	M_r 95,000
Expt 1			
None	—	3.7	20.2
Porcine insulin	3×10^{-10}	12.9	64.0
Porcine insulin	1×10^{-7}	21.5	116.0
Desoctapeptide insulin	1×10^{-7}	4.1	27.2
Expt 2			
None	—	<0.5	5.2
Porcine insulin	1×10^{-7}	4.0	38.2
Multiplication-stimulating activity	1×10^{-7}	<0.5	6.6

Experiments 1 and 2 were performed with solubilized fractions derived from different membrane preparations. The studies were carried out as described in Fig. 1 legend. The autoradiograms were scanned in a Joyce-Loebl microdensitometer and the peak areas corresponding to the 135,000- and 95,000-M_r bands were calculated and expressed in arbitrary units.

of the β subunit of the insulin receptor in intact cells, indicating that this may be an early step in insulin action. This study demonstrates that the same phenomenon can be reproduced in cell-free systems and suggests several interesting points. First, although we are unable to determine the source of the phosphate used to phosphorylate the 95,000-M_r protein in an intact cell, in the broken cell system this protein clearly accepts the phosphate from γ-labelled ATP. Second, the results suggest that an endogenous protein kinase(s) is(are) involved in this reaction and that this endogenous protein kinase activity exists in the plasma membrane fraction and after affinity chromatography on wheat germ agglutinin–agarose. Furthermore, insulin stimulates the incorporation of ^{32}P into the β subunit of insulin receptor at tyrosine residues only. Phosphorylation of tyrosine is rare in normal cells[21], but specific tyrosine phosphorylation of other proteins has been observed after cellular transformation by RNA tumour viruses[21–25] or stimulation of cell growth by epidermal growth factor (EGF)[26,27], platelet-derived growth factor[28] and a human transforming growth factor[29]. Insulin is also a strong growth promoter.

Although phosphorylation has been demonstrated in both the intact and broken cell, there are some differences between them. First, the dose–response curve of phosphorylation is shifted to the left, that is, it is more sensitive, in a cell-free system than in the intact cell. This may depend on several factors, including the conditions of assay and the source of membranes. Second, in the intact cell only the β subunit is phosphorylated, whereas in the cell-free system, both subunits of the insulin receptor are phosphorylated. This suggests the α subunit can also be a substrate for endogenous protein kinases, although the amount of phosphorylation of the α subunit is usually 15% or less than that of the β subunit. The reason we cannot detect the phosphorylation of α subunit in intact cells may be related to the low level of phosphorylation, to the topography of insulin receptor subunits in intact cells, to differences in the level of endogenous phosphorylation, or to differences between these liver membranes derived from normal rats and cultured tumour cells. Third, we have found that in the intact cell, the content of both phosphoserine and phosphotyrosine in the β subunit of the insulin receptor is increased after insulin stimulation[30], although in the intact cell the absolute amount of phosphotyrosine is very small compared with phosphoserine. In the broken cell system, however, only phosphotyrosine is found. These results suggest that the tyrosine phosphorylation of the β subunit may be the initial reaction which occurs on insulin binding and that phosphorylation of serine requires other components absent in cell-free systems. Similar differences of phosphoamino acid composition between intact cells and cell-free systems have been observed in the phosphorylation of RNA tumour virus oncogene products[21,23–25], and the receptor for EGF[26,27].

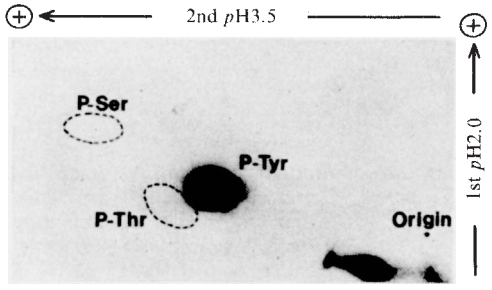

Fig. 2 Identification of phosphoamino acids in the 95,000-M_r phosphoprotein. The phosphoamino acids were analysed by a modification of the method of Hunter and Sefton[21]. The phosphorylation of the 95,000-M_r protein (β subunit of insulin receptor) was stimulated by insulin (10^{-7} M) as shown in Fig. 1. The 95,000-M_r bands, localized by autoradiography, were excised from the gel and the proteins eluted by electrophoresis at 150 V for 20 h into a dialysis bag containing 10 mM sodium phosphate buffer (pH 7.0) and 0.05% SDS. The samples were dialysed against 10 mM NH_4HCO_3, lyophilized, extracted by ethanol/ether (1:1 v/v) and then subjected to acid hydrolysis in 6 M HCl for 2 h at 110 °C. After lyophilization, the hydrolysates were spotted on Whatman 3MM paper. Electrophoresis was performed at pH 2.0 (formic acid/acetic acid/H_2O, 81:25:879) and 550 V for 60 min at 12 °C in the first dimension, and at pH 3.5 (pyridine/acetic acid/H_2O, 1:10:189) and 1,000 V for 105 min in the second dimension. Samples of authentic phosphoserine (Sigma), phosphothreonine (Sigma) and phosphotyrosine (a gift from Dr T. Hunter, Salk Institute) were added to all radioactive samples analysed. The standards were located by ninhydrin and are delineated by the broken lines. The radioactive material was located by autoradiography.

Our results provide the first demonstration, in a cell-free system of a covalent modification of the solubilized insulin receptor as a consequence of insulin–receptor complex formation. The mechanism by which insulin stimulates the incorporation of ^{32}P from [γ-^{32}P]ATP into its own receptor at tyrosine residue is not known. This system does, however, provide the opportunity to investigate whether the insulin receptor is a tyrosine protein kinase, as suggested for the EGF receptor[31], or a regulator of phosphoprotein phosphatase.

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1. Kahn, C. R. *et al. Recent Prog. Horm. Res.* **37,** 477–538 (1981).
2. Czech, M. P. *A. Rev. Biochem.* **46,** 359–384 (1977).
3. Kasuga, M., Karlsson, F. A. & Kahn, C. R. *Science* **215,** 185–187 (1982).
4. Neville, D. M. Jr *Biochim. biophys. Acta* **154,** 540–552 (1968).
5. Freychet, P., Roth, J. & Neville, D. M. Jr *Proc. natn. Acad. Sci. U.S.A.* **68,** 1833–1837 (1971).
6. Cuatrecasas, P. *Proc. natn. Acad. Sci. U.S.A.* **69,** 318–322 (1972).
7. Hedo, J., Harrison, L. C. & Roth, J. *Biochemistry* **20,** 3385–3393 (1981).
8. Kasuga, M., Kahn, C. R., Hedo, J., Van Obberghen, E. & Yamada, K. M. *Proc. natn. Acad. Sci. U.S.A.* **78,** 6917–6921 (1981).
9. Jacobs, S., Hazum, E., Schechter, Y. & Cuatrecasas, P. *Proc. natn. Acad. Sci. U.S.A.* **76,** 4918–4921 (1979).
10. Pilch, P. F. & Czech, M. P. *J. biol. Chem.* **255,** 1722–1731 (1980).
11. Kasuga, M., Hedo, J., Yamada, K. M. & Kahn, C. R. *J. biol. Chem.* (in the press).
12. Rechler, M. M., Podskalny, J. M. & Nissley, S. P. *Nature* **259,** 134–136 (1976).
13. Kasuga, M., Van Obberghen, E., Nissley, S. P. & Rechler, P. *J. biol. Chem.* **256,** 5305–5308 (1981).
14. Avruch, J., Leone, G. R. & Martin, D. B. *J. biol. Chem.* **251,** 1511–1515 (1976).
15. Benjamin, G. J. & Clayton, N. C. *J. biol. Chem.* **253,** 1700–1709 (1978).
16. Seals, J. R., McDonald, J. M. & Jarrett, L. *J. biol. Chem.* **254,** 6991–6996 (1979).
17. Smith, C. J., Wejksnora, P. J., Warner, J. R., Rubin, S. & Rosen, O. M. *Proc. natn. Acad. Sci. U.S.A.* **76,** 2725–2729 (1979).
18. Larner, J. *et al. Science* **206,** 1407–1408 (1979).
19. Marchmont, R. J. & Houslay, M. D. *Nature* **286,** 904–906 (1980).
20. Brownsey, R. W., Belsham, G. J. & Denton, R. M. *FEBS Lett.* **124,** 145–150 (1981).
21. Hunter, T. & Sefton, B. M. *Proc. natn. Acad. Sci. U.S.A.* **77,** 1311–1315 (1980).
22. Witte, O. N., Dasgupta, A. & Baltimore, D. *Nature* **283,** 826–831 (1980).
23. Collet, M. S., Purchio, A. F. & Erikson, R. L. *Nature* **285,** 167–169 (1980).
24. Feldman, R., Hanafusa, T. & Hanafusa, H. *Cell* **22,** 757–765 (1980).
25. Kawai, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **77,** 6199–6203 (1980).
26. Ushiro, H. & Cohen, S. J. *J. biol. Chem.* **255,** 8363–8365 (1980).
27. Hunter, T. & Cooper, J. A. *Cell* **24,** 741–752 (1981).
28. Ek, B., Westermark, B., Wasteson, A. & Heldin, C-H. *Nature* **295,** 419–420 (1982).
29. Reynolds, F. H. Jr, Todaro, G. J., Fryling, C. & Stephenson, J. R. *Nature* **292,** 259–262 (1981).
30. Kasuga, M. *et al. J. biol. Chem.* (in the press).
31. Cohen, S., Carpenter, G. & King, L. Jr *J. biol. Chem.* **255,** 4834–4842 (1980).
32. Laemmli, U. K. *Nature* **227,** 680–685 (1970).

Insertion of diphtheria toxin into and across membranes: role of phosphoinositide asymmetry

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Diphtheria toxin (DT), a 63,000-molecular weight soluble protein, is toxic to most mammalian cells. The mechanism of intoxication involves a step in which one part of the molecule inserts into a membrane, facilitating the transport of the enzymatic fragment of the protein into the cytoplasm[1,2]. This event requires an acidic environment and seems to occur at the membrane of an endocytic vesicle[3,4]. Different cell lines and species differ in their sensitivities to DT—this is due at least in part to differences in the number of DT surface receptors on the cells[5], but may also arise from differences in the membrane transport step. It is generally recognized that protein–lipid

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