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SYNTHESIS OF A CYTOTOXIC INSULIN CROSS-LINKED TO DIPHTHERIA TOXIN FRAGMENT A CAPABLE OF RECOGNIZING INSULIN RECEPTORS

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SUMMARY: Insulin has been cross-linked via a disulfide bond to the diphtheria toxin fragment A which is catalytically active in ADP-ribosylating elongation factor-2 but does not retain binding sites for toxin receptors. The purified conjugate proved to be cytotoxic to mouse Swiss/3T3 cells which are toxin resistant but express insulin receptors. This cytotoxicity coincided with a decrease in protein synthesis and with drastic morphology changes. In contrast, IN-2 cells, which are insulin-nonresponsive variants derived from mouse BALBc/3T3 cells, were resistant to the conjugate. Thus, the conjugate (a chimeric insulin) appears to mediate entry of the toxic fragment A into 3T3 cells through insulin receptors.

INTRODUCTION

The approaches taken, to date, to study the molecular mechanisms by which insulin produces its diverse effects upon cells still leave many questions to be answered. Binding equilibrium and kinetic studies using radioactive and fluorescent probes on intact cells and isolated cell membranes point to a complex mechanism in which only the initial step, the binding to specific cell surface receptors, has been characterized (1,2). It has been proposed that insulin acts while bound to its receptors on the cell surface through an unknown coupling mechanism (3-5). Yet, recent evidence suggests that compartmentalization, internalization and possibly degradation of the hormone or hormone/receptor complex may be necessary for at least some of the insulin-dependent effects (6-11).

We have been undertaking an approach using the strategy of mutagenesis to obtain further insights into the mode of insulin action (12). In an attempt to derive a mutant cell line which is deficient in receptors or receptor-mediated signal transfer mechanisms, we have synthesized a hybrid molecule in which the catalytically active fragment A (M_r 21,000) from diphtheria toxin (13) is covalently cross-linked to a small polypeptide hormone, insulin (M_r 6,000) (Figure 1).

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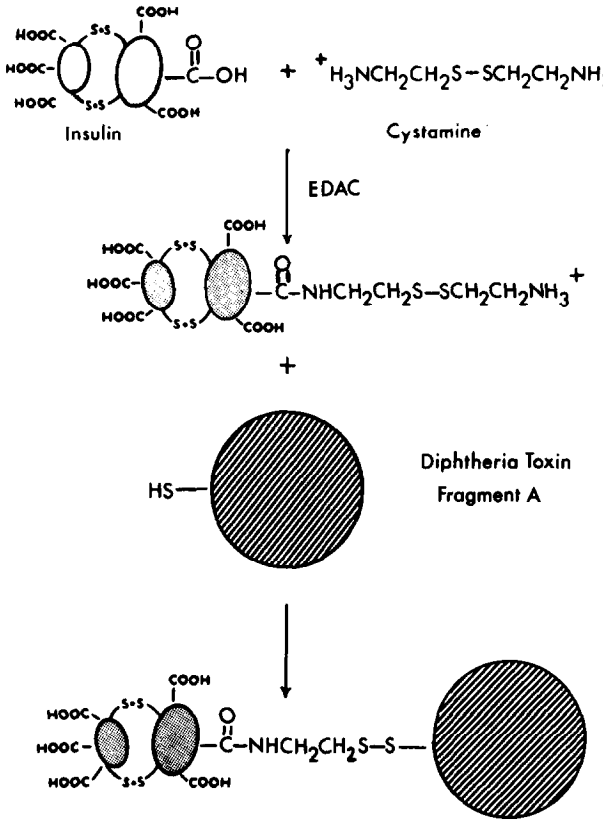

Figure 1. General Scheme for conjugation procedure.

This paper describes the synthesis and preliminary characterization of such a chimeric insulin that is capable of selectively killing insulin receptor-positive cells.

MATERIALS AND METHODS

Preparation of Fragment A. Diphtheria toxin (Connaught Lab., Lot #343) was precipitated by 70% saturated ammonium sulfate, dissolved in a minimum volume of 10 mM Na-phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialyzed toxin (37 mg/ml) was mixed with 2 volumes of 150 mM Tris-HCl, pH 8.0, containing 3 mM EDTA. It was then treated with trypsin (1 µg/ml, Sigma) at 25°C for 30 min. Soybean trypsin inhibitor (Sigma) was added to 1.5 µg/ml to stop the reaction and the nicked toxin was reduced by incubating with 5% 2-mercaptoethanol and 0.1% sodium dodecyl sulfate ($NaDodSO_4$) for 60 min at 25°C. Fragment A was then isolated by the method of Collier and Kandel (14) by column chromatography on Sephadex G-100 (1.5 cm x 110 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.3% $NaDodSO_4$, 1 mM EDTA and 1% 2-mercaptoethanol. The fractions were analyzed by $NaDodSO_4$-polyacrylamide (10%) gel electrophoresis to determine the presence and purity of fragment A by the method of Laemmli (15). Gels were stained with Coomassie brilliant blue (Figure 2-A).

ADP-Ribosylation of Elongation Factor-2 (EF-2). Diphtheria toxin fragment A was assayed for its ability to ADP-ribosylate EF-2 *in vitro* by the method of Moehring and Moehring (16).

Synthesis of Chimeric Insulin. Insulin was cross-linked to toxin fragment A by a modification of the method of Gilliland *et al.* (17) (see Figure 1). Porcine insulin (Eli Lilly, 20 mg) was dissolved in 10 ml of 0.01N HCl and dialyzed against 2 changes of 1.5 liters of 10 mM Na-bicarbonate, pH 7.0, to remove Zn^{+2}. Cystamine dihydrochloride (Sigma, 2.25 mg) was added, dissolved, and the pH was adjusted to 4.7 with 1N HCl. 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (EDAC) (Sigma, 7.67 mg) was added and the pH maintained at 4.7 for 10 min with gentle stirring. The reaction mixture was then dialyzed against 2 changes of 1.5 liters of 20 mM N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES, Sigma), pH 6.5, to remove unreacted cystamine dihydrochloride and EDAC. Diphtheria toxin fragment A (fraction 46 shown in Figure 2-A) was reduced by incubating with 0.1 M dithiothreitol at 4°C for 4 hrs and then dialyzed against 1.5 liters of 20 mM TES, pH 6.5. Approximately 2.5 ml of reduced fragment A (1.0 mg/ml) were added to 10 ml of cystaminyl insulin and the pH was adjusted to 7.4. The mixture was dialyzed against 1.5 liters of 20 mM TES, pH 7.4, overnight at 4°C and then concentrated by membrane cone filtration (Amicon). The conjugate was further purified by a Sephadex G-75 gel filtration (1 cm x 48 cm) equilibrated with 4 M urea and 1 M acetic acid.

Inhibition of Protein Synthesis. To assay amino acid incorporation into polypeptides, media was removed from monolayer cultures, which had been grown to about 70% confluency in Dulbecco's modified Eagle (DME)-medium (GIBCO) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were rinsed with phosphate buffered saline (PBS) and DME-medium without serum was added. The desired amounts of conjugate (Figure 2-B) were added and the cultures were incubated at 37°C for 2 hrs. (Calculation of concentration was based on the assumption that an average of 2 fragment A molecules were conjugated with 1 insulin molecule.) At that time calf serum was added to 10% and incubation was continued for 22 more hrs. The media was removed and the cells were rinsed with PBS. ^{14}C-amino acids (100 µCi/ml, New England Nuclear), which were mixed with minimal essential medium (GIBCO) diluted 20-fold with PBS, were added to the cultures. The cultures were incubated for 1 hr, the media was removed, and the cells were rinsed with PBS and dissolved in 0.5 N NaOH. The ice-cold 5% trichloroacetic acid-insoluble material was collected on a Millipore filter (0.45 µ), dried, and the radioactivity was measured by a Packard scintillation counter.

RESULTS

Synthesis and Purification of Conjugate. The highly pure diphtheria toxin fragment A (fraction 46 in Figure 2-A) retaining its ability to ADP-ribosylate EF-2 was conjugated to insulin as described under Materials and Methods. The conjugate was then fractionated on Sephadex G-75 in which a major peak with a shoulder eluted after the void volume and before the position of toxin fragment A itself which was clearly separated from insulin (elution profile is not shown). The fractions, eluted in the range of molecular weight larger than 27,000 (sum of the molecular weight of toxin fragment A and insulin monomer), were extensively

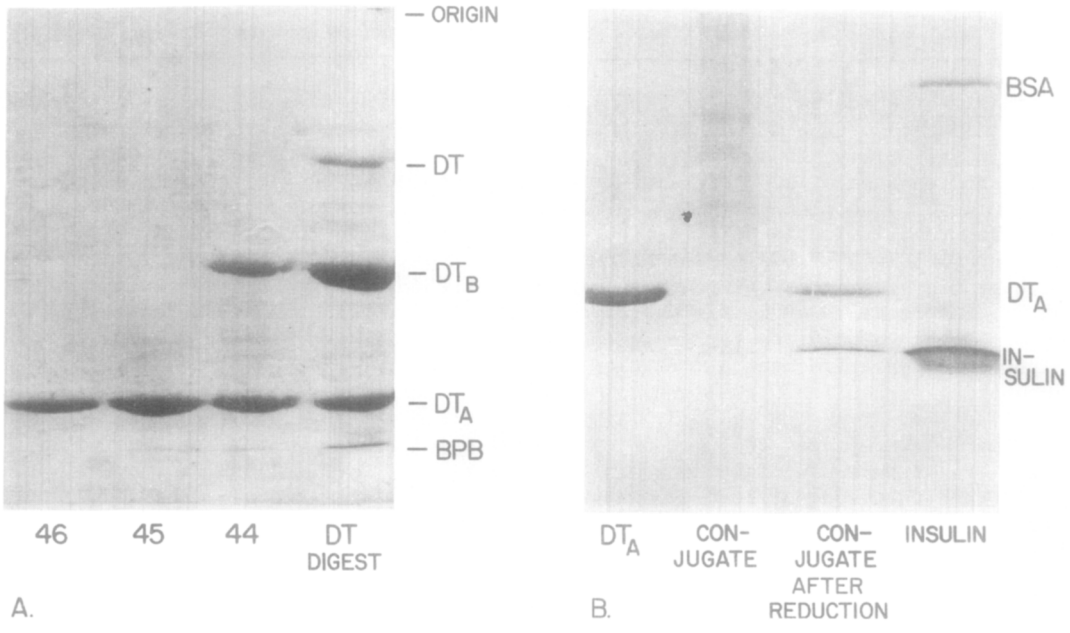

Figure 2-A. $NaDodSO_4$-polyacrylamide (10%) gel electrophoresis of Sephadex G-100 fractions (see Materials and Methods) showing separation and purity of diphtheria toxin fragment A. DT (intact diphtheria toxin); DT_A and DT_B (fragments A and B); BPB (bromophenol blue). The numbers indicate fractions from the Sephadex G-100 column. The electrophoresis conditions were 100 V, 32 mA per gel for 135 min.

Figure 2-B. $NaDodSO_4$-polyacrylamide (10%) gel electrophoresis of conjugation products. The fractions from the Sephadex G-75 column (see Materials and Methods) were analyzed directly or after treating with 2-mercaptoethanol to cleave disulfide linkages. Electrophoresis conditions were the same as in Figure 2-A, except running time (115 min). BSA (bovine serum albumin).

dialyzed against 20 mM TES, pH 7.4. When these fractions were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis (Figure 2-B), 5 bands appeared in the range between fragment A and bovine serum albumin (M_r 68,000). This again indicates the formation of large molecular weight conjugates. We expect to find some heterogeneity of conjugate molecules with slightly different electrophoretic mobilities since insulin contains six carboxyl groups, four of which are thought to be more accessible (18), which could have been modified with cystamine and then cross-linked to fragment A. These conjugate fractions, however, revealed two distinct bands after reduction with 2-mercaptoethanol as is shown in Figure 2-B, one migrating with the toxin fragment A marker and the other migrating with the insulin marker. Thus, disulfide cross-linking between insulin and fragment A is evident.

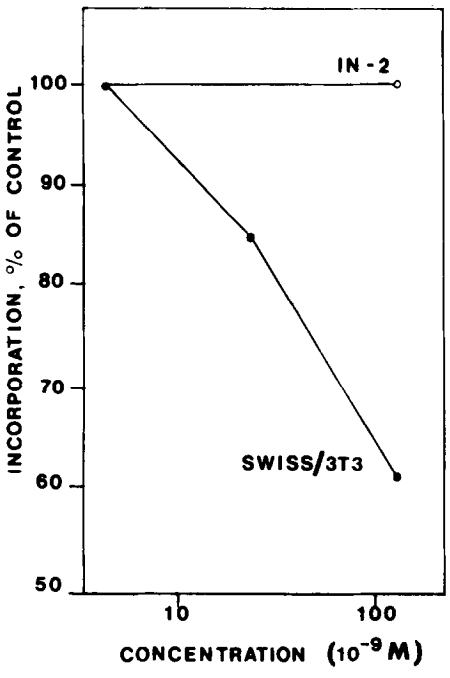

Figure 3. Inhibition of protein synthesis by the insulin-toxin fragment A conjugate in Swiss/3T3 and in IN-2 cells. The IN-2 cell line is an insulin non-responsive variant expressing a small number of insulin receptors with "low binding affinity" (12).

Activity of Conjugate on Cultured Cells. Next, we have tested the biological effects of the conjugate fractions to see if they reveal cytotoxicity to cultured cells and that cytotoxicity can be attributed to the interaction between conjugate (a chimeric insulin) and insulin receptors. Mouse Swiss/3T3 fibroblasts were chosen since they are known to be naturally resistant to intact diphtheria toxin, perhaps due to a deficiency in toxin receptors (13,16,19,20), but are known to express insulin receptors on their cell surface (2,12,21). The EF-2 isolated from these cells is fully sensitive to in vitro modification by intact diphtheria toxin (17). Two parameters were examined for 3T3 cells after treating with the chimeric insulin, effects on protein synthesis and cell viability. As shown in Figure 3, when 3T3 cells were treated with increasing amounts of chimeric insulin and assayed for ^{14}C-amino acid incorporation into polypeptides there was a significant decrease compared to control cultures. Within 24 hrs, Swiss 3T3 cells treated as above showed obvious morphological

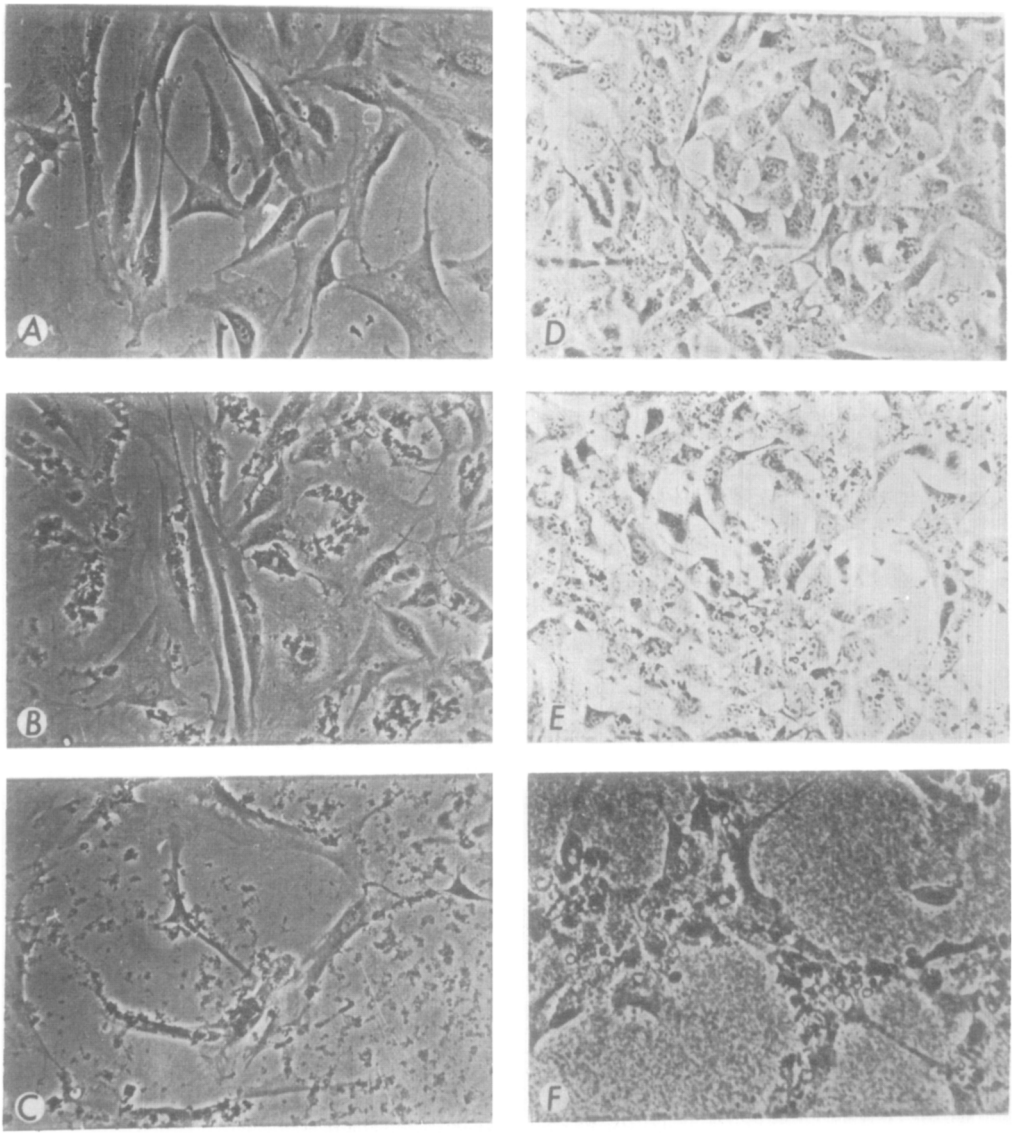

Figure 4. Change of cell morphology. Swiss/3T3 cells were treated with conjugate in the same manner as for assaying protein synthesis except that these were maintained in normal medium afterwards. (A) Control culture at day 0. (B) Cells treated with 1.5 x 10^{-7}M conjugate, at day 1, showing fragmentation. (C) The same culture, at day 2, showing progressive fragmentation. (D) Another control culture at day 3. (E) Cells treated with 6 x 10^{-7}M diphtheria toxin fragment A, at day 3, showing no appreciable morphology change. (F) Cells treated with 9 x 10^{-8}M conjugate, at day 3.

changes (Figure 4). There was apparent bleb formation on the cell surface and cell fragments appeared in the cultures (Figure 4-C and F). The cell destruction continued with further incubation and within 4-5 days all of the cells had died.

No effect was seen in cultures treated with dialysis buffer or with dialyzed material from non-peak fractions from the Sephadex G-75 column. There was also no effect seen in cultures treated with a high dose of toxin fragment A (Figure 4-E) or insulin at 1 x 10^{-6} M (data not shown).

We have tested another mouse cell line, IN-2, which was isolated as an insulin-nonresponsive variant from mutagenized cell populations of mouse BALBc/3T3 fibroblasts and expresses only a small number of insulin receptors with "low binding affinity" (12, and unpublished results). When IN-2 cells were treated in the same manner as Swiss/3T3 cells there was no inhibition of protein synthesis (Figure 3). They grew normally to confluency without showing any apparent change in morphology. Thus, the toxicity of the chimeric insulin seems to require active "high affinity" receptors for insulin.

DISCUSSION

Covalent linkage of cell surface-binding proteins to diphtheria toxin fragment A has been accomplished by others, with varying results. Chang and Neville (22) constructed an artificial hybrid protein with fragment A linked to human placental lactogen. This conjugate failed to show toxicity when tested with organ-cultured mammary gland explants even though the conjugate subunits retained their specific biological activities (23). Gilliland et al. (17) cross-linked Concanavalin A to fragment A. Their conjugate, however did show toxicity in rodents and cultured cells. The data presented in this paper indicate that the small peptide hormone, insulin, moiety of our conjugate seems to recognize insulin receptors and that the entire conjugate or a part containing the toxin fragment A enters into the cytosol through the plasma membrane to exhibit its toxicity. The mechanism of entry of the conjugate may not differ from the endocytotic manner as postulated by Gilliland et al. (17). It is difficult at present to propose the exact role of "high affinity insulin receptors" in promoting entry of the toxin fragment A, but could be related to recent finding of spontaneous cross-linkage between insulin and receptors (8,24). It will then be of great interest to test if the conjugate still delivers the hormonal signal in the metabolic

pathway. For the present study, we have used pure but unfractionated conjugate and perhaps therefore the cytotoxicity was not as effective as intact toxin. Isolation of individual forms of conjugate with different molar ratios of insulin and fragment A and their use would clarify this question. The chimeric insulin has been used in larger scale as a selection agent for mutant cells deficient in insulin receptors and our preliminary data indicate that treatment of mutagenized cell populations of Swiss/3T3 fibroblasts with conjugate has killed a substantial number of cells as expected and that those cells escaping from the first cycle exhibited only 15% of the original insulin binding activity (per cell basis). Repeated treatment will eliminate all the cells except receptor deficient mutants. It may not be unreasonable to expect our chimeric insulin to recognize a new step in the receptor-mediated hormonal signal transfer mechanism.

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