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ASSOCIATION OF THE TRANSFORMED GLUCOCORTICOID RECEPTOR WITH A CYTOSKELETAL PROTEIN COMPLEX

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Summary—In a recent paper we described a system in which glucocorticoid receptors associate with particulate complexes containing tubulin [*Cancer Res.* **49** (1989) 2222s–2229s]. When L cell cytosol is mixed with a microtubule stabilizing buffer and heated to 37°C, the receptor becomes associated with a complex that can be centrifuged out of solution at 150,000 ***g***. In this work we show that the glucocorticoid receptor–cytoskeletal protein complex forms in a temperature and glutamate-dependent manner. Molybdate does not affect generation of the cytoskeletal protein complex but it inhibits association of the receptor with the complex. This suggests that transformation of the receptor to its DNA-binding form is required for interaction with the cytoskeletal complex. Colchicine has no effect on generation of the particulate complex or on the association of receptor with it, suggesting that formation of the complex does not represent a classic *in vitro* process of tubulin polymerization.

INTRODUCTION

In many cells, unliganded glucocorticoid receptors are retained in the cytoplasm until hormone-binding triggers their transport to the nucleus and through the nuclear membrane. Passage through the nuclear membrane is mediated by specific nuclear localization signals, but it is not yet clear what type of system is responsible for transporting these cytosolic proteins to the nuclear membrane or to their sites of action once the nuclear membrane barrier has been passed.

Prior to the 1980s, it was believed that karyophyllic molecules simply diffused to the nucleus and were trapped there by virtue of their affinity for intranuclear components, such as DNA [1]. It later became clear that proteins of mass greater than about 40 kDa require specific signal sequences for nuclear uptake [2]. One of the best characterized of these sequences is the highly basic nuclear localization sequence of the SV40 large T antigen (Pro-Lys-Lys-Lys-Arg-Lys-Val). When this sequence is fused to a normally non-nuclear protein, such as pyruvate kinase, it is itself sufficient to cause efficient nuclear uptake [3]. Nuclear envelope proteins which interact with nuclear localization signals have recently been identified [4, 5] and it is postulated that most large karyophyllic molecules bind to these proteins before being transported into the nucleus by an energy-dependent process.

Using β-galactosidase–receptor fusion proteins, Picard and Yamamoto [6] showed that the glucocorticoid receptor contains two nuclear localization signals. The first signal, NL1 (Thr-Lys-Lys-Lys-Ile-Lys-Gly), is highly homologous to the SV40 sequence. The second signal, NL2, is contained somewhere within the steroid-binding domain and, unlike NL1, its activity in the fusion protein is regulated by hormone-binding. We recently suggested that, once the cytoplasmic glucocorticoid receptor is activated by hormone, it is transferred to the nucleus in an organized way, along a cytoskeletal scaffolding [7]. This suggestion was based on the observation that heating L cell cytosol under tubulin-polymerizing conditions resulted in binding of the receptor to a particulate complex, and, in fact, such microtubule-based transport systems have already been described in neuronal systems [8, 9]. In this paper we demonstrate that it is the transformed state of the glucocorticoid receptor that binds to the cytoskeletal protein complex, which is formed regardless of the presence of colchicine.

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EXPERIMENTAL

Chemicals

BuGR2 monoclonal anti-receptor antibody was provided by Dr R. W. Harrison, III. Monoclonal anti-β-tubulin and goat anti-mouse IgG horseradish peroxidase conjugate antibodies were obtained from Sigma Chemical Co. (St Louis, MO). Immobilon-P transfer membrane was obtained from Millipore Inc. (Bedford, MA).

Cell source and fractionation

L cells were grown in monolayer culture in Dulbecco's modified Eagle medium supplemented with 10% calf serum at 37°C. Cells were harvested by scraping and washed in Earle's balanced saline. The cell pellet was suspended in 1.5 vol of 10 mM HEPES buffer, pH 7.4, 1 mM EDTA, and the cells were ruptured by Dounce homogenization. The homogenate was centrifuged at 100,000 *g* for 1 h, and the supernatant (referred to as cytosol) was used immediately.

Receptor pelleting assay

Aliquots of cytosol (50 μl) were mixed with an equal volume of microtubule stabilizing buffer (20 mM potassium phosphate, 100 mM monosodium glutamate, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.8) or glutamate buffer (10 mM HEPES, 100 mM monosodium glutamate, 1 mM EDTA, pH 7.4) and the mixtures incubated under the conditions indicated in the figure legends. The samples were then centrifuged for 10 min at 150,000 *g* (Beckman Airfuge, 30° angle rotor at 30 psi), and the supernatants were removed and mixed with SDS sample buffer containing 10% β-mercaptoethanol. After rinsing in cold water, the pellets were suspended in SDS sample buffer, and both the supernatant and pellet samples were heated for 2 min in boiling water before electrophoresis.

Gel electrophoresis and immunoblotting

Samples were analyzed by SDS–PAGE using 7% slab gels. M_r standards were: myosin, M_r 205,000; β-galactosidase, M_r 116,000; phosphorylase B, M_r 97,000; bovine serum albumin, M_r 66,000; egg albumin, M_r 45,000; carbonic anhydrase, M_r 29,000. The proteins were then transferred to an Immobilon-P membrane and the membranes were cut horizontally at the 66 kDa marker. The top half was probed with 1% BuGR and the bottom half was probed with 0.2% anti-β-tubulin. Both blots were then probed a second time with peroxidase-conjugated goat anti-mouse IgG and the proteins were visualized using 4-chloronaphthol as the substrate.

RESULTS AND DISCUSSION

In our previous study [7], L cell cytosol was diluted 1:1 (v/v) with a tubulin polymerizing buffer containing phosphate, glutamate, EGTA, and GTP. When this mixture was incubated at 37°C the glucocorticoid receptor was converted from a soluble form to a form that is associated with a particulate complex that pellets at 150,000 *g*. In the experiment shown in Fig. 1, L cell cytosol was diluted in an equal volume of tubulin polymerizing buffer, or in a HEPES buffer with glutamate. As shown in conditions 3 and 4, the presence of glutamate alone is

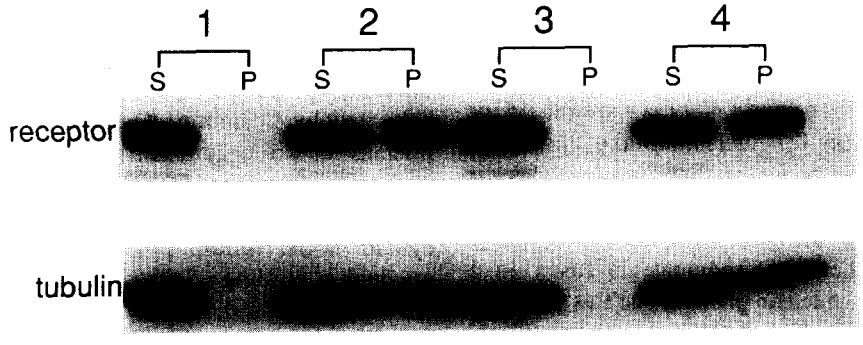

Fig. 1. Pelleting of glucocorticoid receptor and tubulin from L cell cytosol diluted in microtubule stabilizing buffer or in glutamate buffer. Aliquots of L cell cytosol were mixed with an equal volume of microtubule stabilizing buffer or glutamate buffer and incubated at 0 or 37°C for 20 min. Samples were then centrifuged at 150,000 *g*, and the supernatants and pellets were analyzed by SDS–PAGE and immunoblotting as described in the "Experimental" section. The figure shows the peroxidase stain of strips of the immunoblot showing receptor and tubulin present in the supernatants (S) or pellets (P). Incubation conditions were as follows: lane 1, microtubule stabilizing buffer at 0°C; lane 2, microtubule stabilizing buffer at 37°C; lane 3, glutamate buffer at 0°C; and lane 4, glutamate buffer at 37°C.

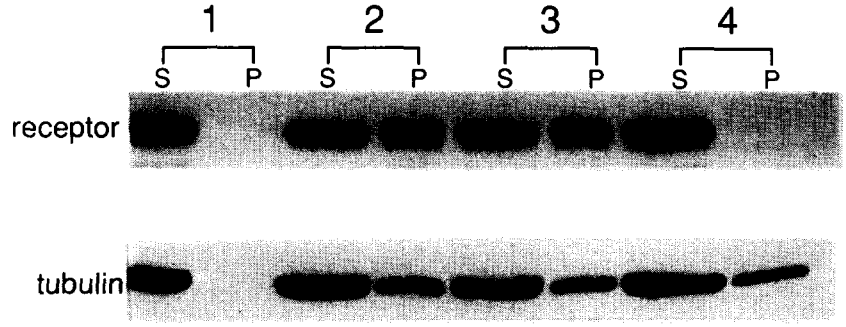

Fig. 2. Effects of colchicine and molybdate on pelleting of receptor and tubulin. Receptor and tubulin pelleting were analyzed as described in Fig. 1 using the following incubation conditions: lane 1, glutamate buffer at 0°C; lane 2, glutamate buffer at 37°C; lane 3, glutamate buffer plus 200 μM colchicine at 37°C; and lane 4, glutamate buffer plus 20 mM molybdate at 37°C.

sufficient to promote the temperature-dependent entry of both tubulin and the glucocorticoid receptor into a complex that is pelleted by centrifugation at 150,000 *g*. This pellet also contains a major portion of vimentin and actin, two other cytoskeletal proteins (data not shown). Pelleting of the receptor and the cytoskeletal proteins does not occur in the absence of glutamate. The fact that conversion of both the receptor and tubulin to a particulate form requires temperature and glutamate, but not magnesium or GTP as is required for polymerization of purified tubulin into microtubules, suggests that the formation of a pelletable complex does not represent a classic *in vitro* tubulin polymerization into microtubules. However, glutamate is known to stabilize microtubules, possibly by facilitating the hydration of tubulin dimers and their interaction with each other [10].

In the experiment in Fig. 2, L cell cytosol was incubated at 37°C in glutamate buffer in the presence of 200 μM colchicine or 20 mM sodium molybdate. From a comparison of conditions 2 and 3 in Fig. 2, it is clear that colchicine has no effect on the pelleting of either tubulin or the receptor. This result also suggests that the pelleted complex may reflect a glutamate-dependent aggregation of tubulin (and other cytoskeletal proteins) and not a classical *in vitro* polymerization process.

When molybdate is added to the pelleting assay (Fig. 2, condition 4) pelleting of the receptor is completely blocked but pelleting of tubulin is unaffected. This suggests that transformation of the receptor to its DNA-binding state, a process that is blocked by molybdate, must occur before the receptor binds to the tubulin-containing particulate. Taken together, this data suggests that the transformed glucocorticoid receptor is the form that interacts with the tubulin-containing complex that is formed in a temperature and glutamate-dependent manner. It is possible that this interaction is somehow related to events that occur during nuclear transfer of the receptor in the cell.

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