serve as a valid initiator. We prefer this second evolutionary scheme but cannot prove that it provides a better explanation of the data than the other. Clearly we cannot exclude the possibility that future information will show the β_2-microglobulin gene to be more closely related to the gene for the constant region of one of the immunoglobulin classes not yet sequenced than it is to the IgG genes.

The two evolutionary schemes considered have in common the hypothesis that the β_2-microglobulin gene was derived from an IgG-like gene. The molecular size and complete amino acid composition of β_2-microglobulin can accordingly be predicted approximately by adding to the presently determined partial sequence of 46 amino acids the remainder of the constant region of IgG_1 from position 390 (where our sequence data end) to the carboxy terminus of IgG_1 at position 446. The predicted values can then be compared with the data of Berggård and Bearn (*5*) observed experimentally. Table 1 shows the comparison. The agreement is satisfactory when allowance is made for the expectation that mutations will have occurred in both descendants subsequent to the first formation of the β_2-microglobulin gene from its presumed IgG-like ancestor. The hypothesis is consequently supported by this independent test. A determination of the complete sequence of β_2-microglobulin should provide a more stringent test.

The data of Berggård and Bearn (*5*) show that the β_2-microglobulin gene is present and translated in normal individuals, since the protein was found in urine and plasma from ten healthy individuals and in five presumed normal samples of cerebrospinal fluid. The questions therefore arise as to whether the DNA corresponding to the beginning part of the immunoglobulin heavy chain gene which we postulate to be the progenitor of the β_2-microglobulin gene is still in the genome of present-day individuals, and if so whether it is transcribed into RNA and translated into protein with or without a variable region attached to it. What function, if any, β_2-microglobulin has in the immune system is likely to depend on the answers to these questions. In any case, a careful search for the function and species distribution of this interesting protein appears to be warranted.

In conclusion, the partial amino acid sequence of a normal human protein, β_2-microglobulin, is compatible with the evolution of its gene from the carboxy terminal portion of an immunoglobulin constant region gene as the result of the use of a new start signal for initiating synthesis of the polypeptide.

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14. The abbreviations used are as follows: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Asx, aspartic acid or asparagine; Cys, cysteine; Cys, cysteine or serine but presumed cysteine from other data; Gln, glutamine; Glu, glutamic acid; Glx, glutamic acid or glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Ser, serine or cysteine but presumed serine from other data; Thr, threonine; Trp, tryptophan; Tyr, tryosine; Val, valine; ?, an unknown residue.
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Cellular Site of Glucocorticoid-Receptor Complex Formation

Abstract. *The cellular site of binding of dexamethasone by specific glucocorticoid receptors in cultured hepatoma cells was investigated with the use of certain mercurials.* p-*Chloromercuribenzene sulfonate and* p-*chloromercuribenzoate inhibit the binding of steroid by receptors in cell-free extracts, but they allow the steroid-receptor complex to form in whole cells. In contrast, $HgCl_2$ inhibits binding both in extracts and cells. Since both organic mercury compounds, unlike $HgCl_2$, do not readily enter intact cells, it appears that the specific steroid binding occurs inside the cell rather than at the cell membrane.*

The first step in the cellular action of steroid hormones in many tissues appears to be intracellular binding of the hormone by cytoplasmic receptors (*1*). The latter, upon complexing with the steroid, are thought to migrate to the nucleus to initiate the characteristic biologic response (*2*). However, many hormones act on the cell surface (*3*). Furthermore, considerable evidence suggests that steroids do interact with and directly modify membranes (*4*) and that, in amphibian oocytes, progesterone can activate protein synthesis by acting on the cell surface (*5*). We therefore thought it important to investigate further, using a different approach, whether the steroid receptors are truly cytoplasmic or merely appear so because they, like many surface proteins (*6*), are released from membranes during cell fractionation.

We have been studying the glucocorticoid-specific receptor interactions in cultured rat hepatoma (HTC) cells, and report here evidence that dexamethasone binding to these receptors takes place inside the cell. For these experiments, we have taken advantage of the findings that mercurials inhibit the specific dexamethasone binding by cytoplasmic extracts of HTC cells (*7*), and that under certain conditions some of these inhibitors do not readily enter

Table 1. Effect of mercurial reagents on specific dexamethasone binding by HTC cells and cell extracts. HTC cells in log phase of growth were harvested by centrifugation at 600*g* for 5 minutes, washed with ice-cold buffer (0.1*M* NaCl, 0.025*M* potassium phosphate, *p*H 7.6), and resuspended in this buffer at 6×10^6 cell/ml. The cell suspension was divided, and from one portion a cytoplasmic extract was prepared (*10*). Either the intact cells or the extracts were exposed to the mercury reagents at 0°C, and after 2.5 minutes (experiment 1) or 5 minutes (experiments 2 and 3) [^{3}H]dexamethasone (2 to $5 \times 10^{-8}M$, 9 to 12 c/mmole, Schwarz), with or without $10^{-6}M$ competing nonradioactive dexamethasone (*10*), was added. The incubations were continued for 5, 10, and 15 minutes in experiments 1, 2, and 3, respectively. After the incubation, the intact cells were collected by centrifugation at 600*g* (5 minutes), and cytoplasmic extracts were prepared from these cells. These extracts, as well as the extracts previously exposed to radioactive steroid, were assayed for specific binding of dexamethasone (*10*). Control values for experiments 1, 2, and 3 for intact cells were 24,175; 14,155; and 16,720 count/min per milliliter, respectively, and for cytoplasmic extracts 1, 2, and 3 were 13,485; 6,610; and 2,367 count/min per milliliter, respectively.

Reagent	Dexamethasone bound (% of control)					
	Intact cell experiments:			Cytoplasmic extracts experiments:		
	1	2	3	1	2	3
None	100	100	100	100	100	100
PCMS ($5 \times 10^{-4}M$)	96	62	75	17	0	0
PCMB ($5 \times 10^{-4}M$)	58	68	55	22	8	0
$HgCl_2$ ($10^{-4}M$)	14	3	0	9	3	0

cells (*8*). We therefore compared the effects of several of these compounds on the formation of the dexamethasone-receptor complex in intact cells and in cell-free extracts. We find that specific binding of dexamethasone by intact cells is inhibited by $HgCl_2$ which readily enters cells, but can occur in the presence of other mercurials that do not easily penetrate cells. In cell-free extracts, formation of the steroid-receptor complex is inhibited by all mercurials tested, at the concentrations used in whole cell experiments.

The results of three experiments are shown in Table 1. The time of exposure of cells or extracts to the inhibitors was kept to a minimum because of the toxic effect of the mercurials. Under these conditions, when cytoplasmic extracts are exposed to either *p*-chloromercuribenzene sulfonate (PCMS), *p*-chloromercuribenzoate (PCMB), or $HgCl_2$, specific binding of dexamethasone is almost completely prevented. When intact cells are exposed to $HgCl_2$, which readily enters cells (*8*), specific dexamethasone binding is likewise prevented. However, when intact cells are exposed to PCMS, which does not easily penetrate cells (*8*), 62 to 96 percent of the specific steroid binding observed in the absence of inhibitor is found. Finally, using PCMB, which enters cells more readily than PCMS but less readily than $HgCl_2$, we observe 55 to 68 percent of the specific steroid binding obtained in the absence of mercurial. Although PCMS and PCMB can inhibit specific steroid binding by whole cells to some extent, their capacity to do so correlates well with their ability to enter cells.

Therefore, differences in mercurial inhibition of specific steroid binding by cells may be explained on the basis of differential penetration of the mercurial into the cell. However, other interpretations should be considered. First, a cell-induced decrease in the concentration or activity of the inhibitors could account for the lack of organic mercurial inhibition of steroid binding by whole cells. This possibility can be excluded since inhibitor-containing media in which cells had been previously incubated retain their ability to block the cell-free binding reaction by 96 to 100 percent. Second, the difference in steroid binding sensitivity to PCMS and PCMB in intact cells and in cell-free extracts could be explained if we assume that the physical state of the receptor is different in cell-free extracts from that in intact cells. This appears unlikely because $HgCl_2$ inhibits steroid-receptor complex formation in both whole cells and cell-free extracts. Finally, the partial inhibition of steroid binding by PCMB and PCMS in intact cells could be explained on the assumption that a proportion of the HTC cell receptors are on the outside surface of the cell. This interpretation can be ruled out because up to 96 percent of the steroid binding observed in control cells was found in the presence of PCMS.

These results indicate that in intact HTC cells the glucocorticoid receptors are relatively insensitive to mercurial reagents that do not readily penetrate cells. This finding, together with earlier observations that binding activity is restricted to the cytosol on cell fractionation, suggests that the receptors are, indeed, intracellular. It remains possible, but unlikely, that the receptors are loosely associated with the membrane in such a way that the mercury-sensitive site is inside the cell and that the steroid-receptor site is on the outside surface. Obviously, the final resolution of this problem requires a far more detailed knowledge of the structure of the cell membrane than is now available.

Since the HTC cell glucocorticoid receptors are probably involved in enzyme induction and in modification of the cell surface (*7, 9, 10*) it is likely that these functions are controlled by steroid action at intracellular sites. These findings do not exclude the possibility that other biological actions of steroids are mediated by surface receptors.

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