Mitogenic agents induce redistribution of transferrin receptors from internal pools to the cell surface

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Incubation of serum-grown HeLa cells in serum-free medium causes a rapid ($t_{1/2}$ 3 min) 30–60% decrease in the binding of ^{125}I -diferric transferrin to the cell surface. Addition of fetal bovine serum to cells in serum-free medium results in a rapid ($t_{1/2}$ 3 min) and concentration-dependent increase in binding activity. The loss or gain in ligand binding is a result of changes in surface receptor number rather than an alteration in ligand–receptor affinity. A variety of hormones (insulin, insulin-like growth factor, interleukin 1 and platelet-derived factor) were found to mimic the effect of serum on receptor number. The alteration in surface receptor number was found to be calcium-dependent. Changes in surface receptor number were independent of either receptor biosynthetic rate or the absolute cellular content of receptors. The effect of insulin or serum on HeLa cell transferrin receptor distribution was unaffected by the presence of transferrin, demonstrating that receptor distribution in this cell type is ligand-independent. The ability of serum or insulin to modify surface transferrin receptor number was also observed in mouse L-cells, human skin fibroblasts, and J774 macrophage tumour cells. However, transferrin receptors on K562 and Epstein–Barr virus-transformed human lymphoblasts were unaltered by these agents. The quantities of receptors whose distribution is predominantly on the surface (i.e. epidermal growth factor or low density lipoprotein receptor) were unaltered by addition of the mitogenic agents. These results extend our previous studies [H. S. Wiley & J. Kaplan (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7456–7460] demonstrating that mitogenic agents can induce redistribution of receptor pools in selected cell types.

INTRODUCTION

Addition of serum to cultured cells induces a variety of biochemical changes, often culminating in mitogenesis. Within minutes, addition of serum to cells causes changes in the activity of a variety of electrolyte and non-electrolyte transport systems, as well as changes in membrane ruffling and cell shape (Moolenaar *et al.*, 1981; Rothenberg *et al.*, 1982; Rozengurt & Heppel, 1975; Schulner & Rozengurt, 1982; Villereal, 1981). The effect of serum on cell behaviour can, for the most part, be ascribed to the presence of hormones or mitogenic agents. While the molecular species in serum which induce these effects have begun to be elucidated, the mechanisms by which these effects alter cellular activities are for the most part unknown.

Recently we demonstrated that addition of epidermal growth factor to cultured human skin fibroblasts (Wiley & Kaplan, 1984), or phorbol esters or calcium ionophores to J774 macrophages tumour cells (Buys *et al.*, 1984; Kaplan *et al.*, 1985), induce a common set of biochemical changes in the respective target cells. These changes include alterations in cell morphology, increased pinocytic activity (Haigler *et al.*, 1979), and increased numbers of surface receptors involved in ligand accumulation (Buys *et al.*, 1984; Wiley & Kaplan, 1984). The increase in surface receptor number results from a redistribution of receptors from internal pools to the cell surface. Most of the intracellular receptors are in the endosome, an organelle involved in receptor recycling

(Buys *et al.*, 1984; Lamb *et al.*, 1983). In this paper we operationally define the endosome as the intracellular reservoir of Tf receptors. For HeLa cells incubated in serum-free medium, 20% of the cellular content of Tf receptors are on the cell surface, 60% are in the endosome and approx. 20% are sequestered from the endocytic apparatus (Lamb *et al.*, 1983). Due to the similarity of the morphological changes induced by the mitogenic agents mentioned above and those induced by serum, we attempted to determine whether serum and other mitogenic agents would also induce translocation of receptors to the cell surface. In this paper we report the characteristics of serum-induced Tf receptor redistributions in cultured cells.

EXPERIMENTAL

Cells

HeLa cells, fibroblasts, and L-cells were grown at 37 °C in a 5% CO_2 incubator in MEM containing Earle's salts with 10% heat-inactivated FBS. J774 cells were grown in a Dulbecco's modification of MEM + 10% FBS. K562 and Epstein–Barr virus-transformed lymphoblasts were grown in RPMI 1640 + 10% FBS. All cells were plated or subcultured at least 2 days before use. Incubations were performed with either Earle's MEM in a 5% CO_2 environment or Hanks MEM + 10 mM-Hepes, pH 7.2, in a room air environment.

Abbreviations used: Tf, transferrin; ^{125}I -Tf(Fe) $_2$, ^{125}I -labelled diferric transferrin; EGF, epidermal growth factor; LDL, low density lipoprotein; PDGF, platelet-derived growth factor; ILGF-1, insulin-like growth factor-1; PBS, phosphate-buffered saline; MEM, minimal essential medium; FBS, fetal bovine serum.

Materials

Human Tf was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.). Insulin (bovine or porcine) was obtained from Eli Lilly (Indianapolis, IN, U.S.A.). PDGF was a gift from Dr. Steve Prescott (University of Utah). LDL was obtained from Dr. Richard Anderson (University of Texas), and EGF was a gift from Dr. Steve Wiley (University of Utah). Rat insulin-like growth factor was a gift from Dr. Gary Smith (University of Nebraska). Deferoxamine (desferrioxamine) was obtained from Ciba-Geigy (Summit, NJ, U.S.A.); aminolaevulinic acid, tin protoporphyrin IX, and haemin were obtained from Porphyrin Products (Logan, UT, U.S.A.); 4,6-dioxoheptanoic acid (succinylacetone) was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.) and ferric ammonium citrate was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Ligands and binding assays

Human Tf was iron-loaded (Ward *et al.*, 1984) and iodinated as described previously (Wiley & Kaplan, 1984). Although expressed in the text as Tf, all preparations of Tf were diferric Tf.

EGF was isolated and iodinated according to the procedure of Wiley & Cunningham (1982). LDL was isolated and iodinated according to the procedure of Goldstein *et al.* (1983). The method of Ward *et al.* (1982) was used to assay binding of ligand to surface receptors. Except where specified, surface receptors were always assayed by addition of ligand to cells at 0 °C. The concentration of ^{125}I -EGF was 20 ng/ml and nonradioactive EGF was 5 $\mu\text{g}/\text{ml}$. The concentration of ^{125}I -LDL was 4 $\mu\text{g}/\text{ml}$ and nonradioactive LDL was 4 mg/ml.

In some experiments ligand bound to surface receptors was assayed at 37 °C. To distinguish between surface-bound and internalized ligand the acid-stripping technique of Haigler *et al.* (1980) was used.

In order to measure ^{125}I -Tf binding to receptors which were occupied by non-radioactive Tf the following technique was employed. After being exposed to unlabelled Tf, cells were incubated with 1.5 ml of 0.01 M-citric acid/0.02 M- Na_2HPO_4 /0.15 M- NaCl /0.8 mM-deferoxamine, pH 5.4, for 1.5 min at 0 °C. The solution was then removed and cells were incubated in PBS containing deferoxamine at 0 °C for an additional 3 min. This procedure was repeated three times, the cells were then washed twice with cold PBS, and ligand-binding activity was determined.

Additional procedures

Protein determinations were performed as described by Lowry *et al.* (1951) using bovine serum albumin (fraction V, Sigma) as a standard.

RESULTS

Effect of serum on surface Tf receptor number in HeLa cells

Removal of serum from HeLa cells grown in serum-containing medium results in a decrease in surface Tf receptors (Fig. 1a). The kinetics of the decrease suggested a biphasic response: a rapid decrease with a half-time of 3 min and a slower response resulting in a continued decline in ligand binding activity over a time span of hours (results not shown). The magnitude of the rapid loss was 30–60% of the ligand-binding activity seen in serum-grown cells. The results in this paper are solely concerned with the rapid response.

While incubation of cells in serum-free medium resulted in a decrease in receptor number, addition of serum to cells in serum-free medium resulted in a rapid reappearance of surface binding activity (Fig. 1b). The rate of appearance of surface binding activity suggests monophasic kinetics, indicating that the rapidly respond-

Fig. 1. Effect of serum withdrawal (a) or addition (b) on HeLa cell Tf receptors

(a) Cells were grown in MEM + 10% FBS for 4 days. Cells were then washed and incubated in serum free MEM + 10 mM-Hepes and incubated at 37 °C or 0 °C. At specified times, cells incubated at 37 °C were placed on ice and washed twice with cold PBS, and surface binding was determined. ●, Cells incubated at 0 °C in serum-free MEM; ○, cells incubated at 37 °C in serum-free MEM. (b) Cells were grown in MEM + 10% FBS for 4 days. Cells were then changed to serum-free medium and placed at 0 °C or 37 °C for 30 min. At the end of this incubation samples were assayed for specific binding, or fetal calf serum (final concentration 10%) was added to the remaining samples at 37 °C. At specified times samples were placed at 0 °C, washed, and specific binding in samples incubated at 37 °C (●) or at 0 °C (○) was determined. The inset represents linearization of the data.

Fig. 2. Analysis of the effect of serum removal on the concentration dependency of ^{125}I -Tf binding to HeLa cells

Cells were incubated in serum-containing medium or in serum-free medium for 60 min at 37 °C. All cells were then placed at 0 °C and washed twice with cold PBS. The cells were then incubated with different concentrations of ^{125}I -Tf for 60 min, and specific binding in cells incubated with serum (●) or without serum (○) was determined. The data were analysed according to the method of Scatchard (1949).

ing fraction of Tf receptors behaves homogeneously. The serum-dependent alterations in ligand binding activity were due to changes in receptor number rather than changes in ligand-receptor affinity (Fig. 2). Examination of the concentration dependency of ^{125}I -Tf binding to cells incubated in either serum-containing or serum-free medium demonstrated identical ligand affinity (K_d 10^{-8} M).

The serum-induced increase in receptor number was concentration-dependent (Fig. 3). Maximal increases in receptor number were observed within 15 min of addition at a serum concentration of 10%. At this concentration there was a marked 'overshoot' of receptor number relative to cells continuously maintained in serum. If receptor number was assayed 4 h after serum addition, a slightly lower absolute value was obtained, suggesting a time-dependent decrement in the observed response. The amount of the overshoot and the exact serum concentration at which the maximal response occurred varied slightly from experiment to experiment. However, the increase in receptors was always concentration-dependent and was dependent upon addition of the mitogen to cells at 37 °C. That is, incubation of cells with serum at 0 °C did not lead to any change in receptor number.

Total receptor content in HeLa cells varied depending on growth rate (Ward *et al.*, 1982). Therefore, changes of surface receptor numbers were always compared with control cells used on the same day. As demonstrated below, changes in the distribution at receptors between the cell surface and internal pools was independent of absolute receptor numbers.

Fig. 3. Effect of different concentrations of FBS on HeLa surface Tf receptor number

Cells grown in serum-containing medium (●) were incubated in serum-free medium for 60 min at 37 °C. Different concentrations of serum were added to the cells for 15 min (○) or 4 h (Δ). At the end of the specified incubation periods cells were placed at 0 °C and specific binding was determined.

Effects of serum and hormones on Tf-receptor number

For all of the above experiments we utilized FBS since bovine Tf does not measurably interact with human Tf receptors (Ward *et al.*, 1982). We next addressed the question of whether the effect of serum addition or withdrawal was specific to bovine serum albumin. The major difficulty in examining whether sera from other species would affect Tf receptor number is that most animal Tf exhibits measurable affinity for the human Tf receptor. Binding of xenogeneic Tf to Tf receptors gives rise to two problems. The first is that a procedure is required to measure ligand binding to receptors which may be occupied by Tf present in the serum. The second problem is that Tf itself may induce redistribution of receptors which may obviate or obscure the serum effect. To eliminate these problems we devised a technique to assay occupied receptors. This was accomplished by modifying the acid-stripping procedure of Haigler *et al.* (1980) such that Tf could be removed from surface Tf receptors without affecting their ability to rebind ^{125}I -Tf. This protocol involved incubation of cells at low pH in the presence of chelating agents to remove Fe from Tf followed by incubation at neutral pH to allow apotransferrin to dissociate from receptors. This protocol mimics the cellular Tf cycle (Daultry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Exposure of cells incubated at 0 °C with ^{125}I -Tf to three cycles of the acid-stripping treatment resulted in the removal of 80–90% of receptor-bound radioactivity (results not shown). Increasing the number of cycles from three to four had no further effect on removal of cellular radioactivity. In a number of independent experiments, we observed a 10–17% reduction in binding activity. While on an

absolute level this reduction is small, the magnitude of the serum effect was 30–60%, and thus this decrement may constitute a significant proportion of this response. To circumvent this problem, we incubated all cells at 0 °C with Tf prior to treatment with the acid/chelating solutions and then assayed for surface Tf receptors. By exposing all cells to Tf at 0 °C, we essentially normalize for this variable. Using the acid-stripping protocol, we could then determine whether the effect of serum was restricted solely to sera of bovine origin. As demonstrated in Table 1, human serum (either its presence or absence) affects surface Tf receptor numbers in a manner similar to bovine sera (either fetal, newborn, or calf). Receptor distribution remained similar in cells incubated at 37 °C in the presence or absence of Tf (results not shown) indicating that in this cell type receptor distribution is unaffected by ligand binding.

Effect of hormones on surface Tf-receptor number

Serum as it is generally prepared contains growth-promoting activities derived in part from the platelet release reaction, PDGF being the best studied of the platelet growth factors. To determine whether the serum effect could be ascribed to such factors we examined the effect of plasma on Tf receptor number. Addition of either bovine or human plasma to HeLa cells incubated in serum-free medium resulted in an essentially complete recovery of Tf receptor number. These results suggest that platelet-derived activities or products of plasma cascade reaction (fibrinopeptides, kinins, or complement) cannot be responsible for changes in receptor activity on HeLa cells. Addition of purified PDGF to HeLa cells did give rise to a small increase in surface receptors. Fibroblasts showed a much higher increase in surface receptors when exposed to PDGF but only at concentrations of 10^{-7} M and higher (Fig. 4). These concentrations are probably too high to be of physiological significance.

In considering what factors in serum or plasma might be responsible for the change in surface receptor number, we were drawn to the observation that insulin could induce membrane ruffling in fibroblasts (Goshima *et al.*, 1984) and vesicle translocation in adipocytes (Kono

Fig. 4. Effect of different concentrations of hormones on human fibroblast or HeLa cell surface Tf receptors

Cells grown in serum-containing medium were incubated in serum-free medium for 60 min at 37 °C. Samples were then placed at 0 °C for determination of specific binding (control). Different concentrations of hormones [insulin (○), IgGF-1 (●) or PDGF (△)] were added to cells incubated in serum-free medium. The cells were then incubated at 37 °C for 15 min and placed at 0 °C for determination of specific binding. (a) Fibroblasts, (b) HeLa cells.

et al., 1982). The data in Fig. 4 demonstrate that incubation of either human skin fibroblasts or HeLa cells with insulin induces, in a concentration-dependent manner, alterations in surface Tf receptor number.

The concentration of insulin which causes a half-maximal increase (ED_{50}) in receptor number is 10^{-7} M. The effect of insulin at this concentration may be due to

Table 1. Effect of different types of serum or plasma on HeLa cell surface Tf receptor number

Cells grown in serum-containing medium were washed twice with cold PBS and placed at 0 °C for determination of specific binding (column 1). Other samples were washed and placed in serum-free medium for 60 min at 37 °C, at which point samples were taken for measurement of specific binding (column 2). Human or bovine plasma or serum (final concentration 10%) was added for 30 min at 37 °C to cells remaining in serum-free medium prior to determination of specific binding (column 3). To measure specific binding all samples were incubated with 5×10^{-8} M-Tf(Fe)₂ for 60 min at 0 °C, and then washed with citric acid/deferoxamine solution as described in the Experimental section prior to determination of specific surface binding. Values are $\pm$ S.E.M.

		Specific activity (fmol of ¹²⁵ I-Tf/μg of protein)		
		1 Control	2 – Foetal calf serum	3 + Serum or plasma
Expt. (a)	FBS	2.50 ± 0.10	1.33 ± 0.07	2.26 ± 0.07
	Human serum	–	–	2.21 ± 0.07
	Bovine plasma	–	–	1.97 ± 0.04
Expt. (b)	FBS	3.11 ± 0.14	1.08 ± 0.08	2.77 ± 0.10
	Human serum	–	–	2.70 ± 0.23
	Human plasma	–	–	3.01 ± 0.03

the interaction of insulin with insulin-like growth factor receptors. To test this possibility we examined the effect of purified rat ILGF-1 on surface Tf receptor numbers. As shown in Fig. 4, ILGF-1 also increased surface receptor number in both HeLa cells and fibroblasts. Purified ILGF-1 was capable of altering surface receptor number. However, there did not appear to be any difference in efficacy between ILGF-1 and insulin. In preliminary studies we have also observed that purified preparations of interleukin-1 increased surface Tf number in fibroblasts (S. Rockwood, unpublished work).

The change in surface Tf receptor numbers is independent of Tf receptor biosynthetic rate

The rapidity of the change in surface Tf receptor number upon addition or withdrawal of serum strongly suggests that changes in surface receptor number are independent of Tf receptor biosynthesis. Our previous studies suggested that HeLa cell Tf receptor number was regulated by the level of intracellular haem and that regulation of Tf receptors occurred at the level of receptor biosynthesis (Ward *et al.*, 1982, 1984). Cells with increased numbers of Tf receptors were obtained by incubating cells with succinylacetone, an inhibitor of aminolaevulinic acid dehydrase (an enzyme involved in porphyrin biosynthesis), or deferoxamine mesylate, a chelator of intracellular iron. Cells with reduced numbers of receptors were obtained by incubating cells with aminolaevulinic acid, thus bypassing the rate-limiting step in haem biosynthesis, aminolaevulinic acid synthetase. Reduced numbers of receptors also resulted from prolonged incubation with either ferric ammonium citrate, haemin, or tin protoporphyrin IX. Some of these agents (succinylacetone or aminolaevulinic acid) work most effectively in serum-free media. In these cases we measured only the effect of serum addition on surface receptor number. Cells preincubated in cycloheximide (5 µg/ml) showed marked increases in the number of surface receptors upon exposure to serum. Alteration in

surface receptor number due to the presence or absence of serum was unaffected by the total number of cellular Tf receptors (Table 2). Addition of cycloheximide to cells at concentrations that inhibit both amino acid incorporation and Tf receptor biosynthesis (Ward *et al.*, 1982) did not prevent the serum-induced increase in surface receptor number. These results indicate that manipulation of surface receptor number by serum is independent of either the biosynthetic rate and/or absolute cellular content of receptors.

Effect of serum on Tf receptor number in other cell types

The effect of serum on surface Tf receptor number is not restricted to HeLa cells. As shown in Table 3, human skin fibroblasts, J774 tumour cells (a cell line exhibiting macrophage characteristics) and L929 (mouse fibroblasts) also increased their surface Tf receptor number in response to the addition of serum. In these experiments cells were also incubated at 0 °C with bovine serum to determine whether bovine Tf exhibited any interaction with surface Tf receptors. The data demonstrate that addition or withdrawal of serum from cells incubated at 0 °C did not effect binding of ¹²⁵I-Tf. Human Epstein-Barr virus-transformed lymphoblasts or K562 cells did not exhibit any alteration in Tf surface receptor number in response to either serum addition, serum removal, or to addition of insulin (Fig. 4). This observation demonstrates that the effects of serum on Tf receptor activity are not ubiquitous, and exhibit a cell or tissue specificity.

Effect of serum and insulin on other surface receptors

Our previous studies indicated that the induced increase in surface Tf receptors was the result of translocation of internal pools to the cell surfaces (Buys *et al.*, 1984; Wiley & Kaplan, 1984). A corollary to this observation is that plasma membrane molecules that are not present in intracellular pools would be little altered by these agents. This point was examined by measuring the effect of serum, insulin or the calcium ionophore A23187 on surface receptors for Tf, LDL, or EGF.

Table 2. The effect of serum addition withdrawal on receptor distribution is independent of absolute receptor content

HeLa cells were grown in serum-containing medium for 2 days. The cells were washed once with PBS and then placed in either MEM or MEM with 10% FBS. The cells were then incubated for 72 h in the specified concentrations of reagents, or for 1 h with cycloheximide. At the end of the incubation period cells that had been maintained in serum-containing medium were washed with PBS and placed in serum-free medium for 30 min at 37 °C. Alternatively, FBS (final concentration 10%) was added (30 min, 37 °C) to cultures that had been maintained for 72 h or 1 h in serum-free medium. At the end of the incubation cells were placed at 0 °C and specific binding determined. Values are $\pm$ S.E.M.

Reagent	Specific activity (fmol of ¹²⁵ I-Tf/µg of protein)			
	Cells incubated in serum-containing medium		Cells incubated in serum-free medium	
	Control	– FBS	Control	+ FBS
None	1.28 $\pm$ 0.08	0.87 $\pm$ 0.03	0.75 $\pm$ 0.01	1.36 $\pm$ 0.12
Aminolaevulinic acid (0.25 mM)	–	–	0.38 $\pm$ 0.01	0.85 $\pm$ 0.10
Succinylacetone (0.10 mM)	–	–	1.27 $\pm$ 0.03	1.82 $\pm$ 0.01
Tin protoporphyrin IX (14 µg/ml)	–	–	0.48 $\pm$ 0.02	1.07 $\pm$ 0.05
Deferoxamine (0.05 mM)	3.33 $\pm$ 0.21	2.29 $\pm$ 0.07	3.06 $\pm$ 0.16	4.48 $\pm$ 0.25
Ferric ammonium citrate (2.81 mg/ml)	0.52 $\pm$ 0.01	0.25 $\pm$ 0.01	0.35 $\pm$ 0.02	0.71 $\pm$ 0.02
Haemin (0.1 mM)	0.47 $\pm$ 0.01	0.23 $\pm$ 0.03	0.59 $\pm$ 0.03	1.05 $\pm$ 0.08
Cycloheximide (5 µg/ml)	–	–	0.81 $\pm$ 0.03	1.64 $\pm$ 0.12

Table 3. Response of different cell types to serum withdrawal

Cells were grown for 2 days in ^aEarles' MEM + 10% FBS, ^bDulbecco's MEM + 10% FBS, or ^cRPMI 1640 + 10% FBS. The cells were either placed at 0 °C or maintained at 37 °C and incubated with serum-containing or serum-free media for 60 min. All samples were then placed at 0 °C, washed twice with PBS, and specific binding was determined. Values are $\pm$ S.E.M.

Cell type	Specific activity (fmol of ¹²⁵ I-Tf/ μ g of protein)			
	0 °C		37 °C	
	+ FBS	– FBS	+ FBS	– FBS
HeLa ^a	4.26 $\pm$ 0.03	4.05 $\pm$ 0.13	4.01 $\pm$ 0.13	1.81 $\pm$ 0.09
J774 ^b	4.29 $\pm$ 0.24	4.13 $\pm$ 0.13	4.38 $\pm$ 0.15	1.33 $\pm$ 0.08
SW8 ^a	0.79 $\pm$ 0.02	0.88 $\pm$ 0.03	0.85 $\pm$ 0.02	0.41 $\pm$ 0.02
L cells ^a	0.21 $\pm$ 0.02	0.21 $\pm$ 0.03	0.23 $\pm$ 0.01	0.12 $\pm$ 0.01
K562 ^c	0.98 $\pm$ 0.01	0.97 $\pm$ 0.02	0.97 $\pm$ 0.01	0.97 $\pm$ 0.05
EBV-lymphoblast ^c	0.27 $\pm$ 0.01	0.27 $\pm$ 0.01	0.28 $\pm$ 0.02	0.28 $\pm$ 0.01

Studies to date indicate that in cells not exposed to ligand the bulk of LDL (Basu *et al.*, 1981) and EGF (Wiley & Cunningham, 1982) receptors are located on the plasma membrane. As demonstrated in Table 4, addition or removal of mitogenic agents only modified TF receptor number and had no effect on either EGF or LDL binding activity. The results suggest that membrane receptors behave heterogeneously and that the effects of these agents are probably directed at membrane receptors which exhibit a significant intracellular localization.

Effect of serum and insulin on surface receptor number requires extracellular divalent cations

Studies have demonstrated that diverse mitogenic agents have similar effects on cells. One of the earliest of metabolic responses is increased intracellular Ca²⁺ (Hesketh *et al.*, 1985). The increase in intracellular Ca²⁺ is dependent on the presence of extracellular Ca²⁺. Based on these observations we tested whether the hormone-induced receptor redistribution was also Ca²⁺-dependent. Cells were incubated in either Ca²⁺-containing or Ca²⁺-free medium, and the effect of serum or insulin on surface receptor number was determined. As demonstrated in Table 5, the effect of these agents required extracellular Ca²⁺. This observation suggests that the increase in surface receptor number is one of the common early responses of these cells to mitogenic agents.

DISCUSSION

The results in this paper extend our previous studies on receptor redistribution (Buys *et al.*, 1984; Kaplan *et al.*, 1985; Wiley & Kaplan, 1984). In those studies we demonstrated that pharmacological agents such as calcium ionophores, thiol treatment or the mitogenic hormone EGF could induce the translocation of intracellular compartments to the cell surface. The compartment that appeared most altered was the endocytic apparatus and increases in surface receptors occurred with a concomitant decrease in the endosomal content of receptors.

The results of the present study demonstrate that addition or removal of serum changes the number of surface Tf receptors in a variety of cell types. Incubation of serum-grown cells in serum-free medium results in a

Table 4. Effect of mitogenic agents on HeLa cell Tf, LDL, and EGF receptors

(a) HeLa cells grown in serum-containing medium for 3 days were either changed to fresh medium + 10% FBS or changed to serum-free medium. Cells were then incubated at 37 °C for 60 min, placed on ice, and specific binding of either ¹²⁵I-Tf or ¹²⁵I-EGF were determined. Values are $\pm$ S.E.M.; (1) and (2) denote separate experiments. (b) HeLa cells were grown in serum-containing medium for 3 days. Cells were then incubated in serum-free medium for 18 h. (This time period allows for derepression of LDL receptor biosynthesis.) Samples were taken for specific binding of either Tf or LDL. Insulin (10⁻⁶ M) was added to the cultures for 15 min at 37 °C and then specific binding was determined at 0 °C. Values are $\pm$ S.E.M.; (3) and (4) denote separate experiments.

Receptors assayed		Specific activity (fmol of Tf or EGF/ μ g of protein or ng of LDL/ μ g of protein)	
		+ Serum	– Serum
(a)			
Tf	(1)	2.66 $\pm$ 0.06	1.42 $\pm$ 0.12
	(2)	2.09 $\pm$ 0.10	1.30 $\pm$ 0.03
EGF	(1)	0.27 $\pm$ 0.01	0.25 $\pm$ 0.01
	(2)	0.29 $\pm$ 0.02	0.30 $\pm$ 0.02
		– Insulin	+ Insulin
(b)			
Tf	(3)	2.38 $\pm$ 0.05	3.74 $\pm$ 0.19
	(4)	1.38 $\pm$ 0.05	2.56 $\pm$ 0.08
LDL	(3)	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01
	(4)	0.10 $\pm$ 0.01	0.09 $\pm$ 0.01

precipitous drop in surface Tf receptor number, whereas addition of serum to cells in serum-free medium results in a similarly rapid increase in receptor number. The rapidity of the response as well as its reversibility suggest that changes in receptor number are independent of receptor biosynthesis or degradation. This point was directly addressed by demonstrating that incubation of cells in the presence of agents which increase or decrease absolute receptor number did not affect the relative

Table 5. Effect of divalent cations on observed alterations in surface Tf binding

Cells grown in serum-containing medium were washed twice and placed in serum-free medium for 60 min at 37 °C. Cells were then placed in medium with or without Ca^{2+} + Mg^{2+} . Serum (10%) or insulin (10^{-6} M) was added and cells were incubated at 37 °C for 15 min. All samples were then placed at 0 °C, washed with Ca^{2+} + Mg^{2+} -containing medium and specific binding was determined. Values are $\pm$ S.E.M.

Additive	Ca^{2+} + Mg^{2+}	Binding activity (%)
–FBS	+	100 $\pm$ 4.0
+FBS	+	183 $\pm$ 11.7
+FBS	–	110 $\pm$ 5.2
+Insulin	+	183 $\pm$ 12.6
+Insulin	–	109 $\pm$ 7.4

magnitude of the observed results of serum addition or removal (Table 1). The kinetics of receptor appearance resulting from serum addition suggested a single kinetic component with a half-time for receptor appearance of 3–5 min. The half-time for receptor appearance due to biosynthesis is approx. 14–17 h (Ward *et al.*, 1982), further suggesting that the observed changes are not due to alterations in turnover rate.

The effect of serum and insulin on Tf receptor number is not unique to HeLa cells. Mitogen-dependent alterations in surface receptor number were also observed in human skin fibroblasts, mouse macrophage tumour cells and mouse L cells. Changes in surface Tf receptors were not restricted to bovine serum. Human serum or plasma were equally effective in altering surface receptor number. In an attempt to delineate the component(s) in serum which is responsible for these changes, we focused on the study of Barnes & Sato (1980) who demonstrated that HeLa cells could grow in serum-free medium if supplemented with insulin, and the study of Goshima *et al.* (1984) which demonstrated that insulin could affect membrane ruffling and amino acid transport. As demonstrated in Fig. 4, addition of insulin to cells incubated in serum-free medium restored surface receptor number. Half maximal concentration was 10^{-7} M, and at higher concentrations, there appeared to be an overshoot phenomena such that supranormal levels of surface receptors were observed at times immediately after insulin addition. At later time points receptor number declined to values seen in cells incubated continuously in serum-containing medium.

The data in Table 4 demonstrate that, while serum and insulin altered Tf receptor number, these agents had little effect on EGF or LDL receptors. Independent lines of evidence indicate that, in the absence of ligand, the majority of cellular LDL (Basu *et al.*, 1981) or EGF (Wiley & Cunningham, 1982) receptors are on the cell surface. Alternatively, receptors that are present in substantial quantities in intracellular pools (i.e. α -macroglobulin–proteinase complexes or mannose-terminal glycoproteins in alveolar macrophages) may be redistributed upon exposure to secretagogues (Buys *et al.*, 1984). These observations suggest that assessing alteration in surface receptor number upon secretagogue administration may provide a useful approach to

determining the subcellular distribution of receptors. In HeLa cells Tf receptors are constitutively recycled regardless of whether Tf is present (R. Ajioka & J. Kaplan, in the press). However, studies indicate that in other cells the internalization of the Tf receptor may be ligand-dependent or that the rate of internalization of occupied receptors may be different from that of unoccupied receptors (May *et al.*, 1984; Klausner *et al.*, 1984). Although the conclusions of some of these studies have recently been questioned (Watts, 1985) preliminary data in our laboratory suggest that in some cell types receptor internalization and consequently receptor distribution may be ligand-dependent. In these cell types (human normoblasts or reticulocytes, HL60 cells, and Epstein–Barr virus-transformed human B-lymphoblasts) the majority of Tf receptors are on the cell surface with only minor amounts present in the internal compartment. It would thus stand to reason that serum or insulin would have little effect on changing surface receptor number, in part because of the small internal receptor pool. These observations would also suggest that the effect of these agents are on internalization rate rather than exteriorization from an internal pool. Studies are necessary in an attempt to determine what factors specify the distribution of cellular Tf receptors.

The results presented in this and our previous studies demonstrate that receptor translocation is an early response of cells to mitogenic agents (serum, insulin, EGF). Vesicle–cell-surface fusion has been suggested as a mechanism by which transport of electrolytes or metabolites may be regulated (Leinhardt, 1981). If the endosome was enriched in transporters then translocation of endosomal components to the cell surface would place these components in an environment that would allow them to function. The ability of insulin to induce redistribution of glucose transporters in adipocytes is consistent with this hypothesis (Kono *et al.*, 1982). To prove this hypothesis, it will be necessary to determine whether other transport activities are located in vesicles and whether their distribution can be affected by the appropriate secretagogues.

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