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STIMULATION OF THE RELEASE OF TWO GLYCOPROTEINS FROM MOUSE 3T3 CELLS BY GROWTH FACTORS AND BY AGENTS THAT INCREASE INTRALYSOSOMAL pH

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SUMMARY: Peptide growth factors selectively increase the amount of mitogen-regulated protein (MRP) and major excreted protein (MEP) released by mouse 3T3 cells. Balb/c 3T3 cells release mainly MEP and Swiss 3T3 cells release mainly MRP. Fibroblast growth factor, epidermal growth factor, nerve growth factor, serum, and concanavalin A increase the extracellular appearance of both MEP and MRP, but to different extents. Several agents that have been shown to, or would be expected to increase, intralysosomal pH also selectively increase the release of MEP and MRP from both Balb/c and Swiss 3T3 cells. The effective agents are monensin, nigericin, ammonium chloride, methylamine, chloroquine, and high extracellular pH.

INTRODUCTION

We have shown that growth factors, such as EGF* and FGF, selectively stimulate the synthesis and release of a group of glycosylated proteins with a common polypeptide core (1). These "mitogen-regulated proteins" (MRP, formerly called mitogen-releasable proteins [1]) are released in abundance by Swiss 3T3 cells in response to the growth factors and in lesser amounts by human fibroblasts and Balb/c 3T3 cells. We also found that the synthesis and release of another protein is selectively increased by FGF (1). This protein, "major excreted protein" (MEP), was first described by Gottesman as being increased in the medium of cells transformed by Kirsten or Moloney sarcoma viruses (2). The synthesis and release of MRP, on the other hand, is decreased after transformation by SV40 or Moloney sarcoma virus (1).

Previously we have shown that stimulation by growth factors of the appearance of extracellular MRP is mediated by an actinomycin D-sensitive step and that mitogens increase the extracellular amount of both the glycosylated and nonglycosylated forms of MRP (1). To further characterize the intracellular functions that regulate the extracellular concentration of MRP,

* Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; MRP, mitogen-regulated proteins; MEP, major excreted protein; 3T3 cells, mouse embryo fibroblasts; NGF, nerve growth factor; Con A, concanavalin A.

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we investigated the effect of agents that inhibit lysosomal protein degradation by increasing intralysosomal pH. Here we report that the amount of extracellular MRP and MEP is selectively increased both by protein mitogens and by agents that have been shown to increase intralysosomal pH, or which are likely to increase the intralysosomal pH.

METHODS

Cell Culture. Stock cultures of Swiss 3T3-4a and Balb/c 3T3A31 cells were kept in Dulbecco-Vogt's modified Eagle's medium (DME), supplemented with 10% calf serum. The stock cell cultures were grown as monolayers on plastic, Petri tissue-culture dishes, at 37°C, in a water-saturated atmosphere, with 10% CO_2 in air. Monthly checks for mycoplasma were made by autoradiography or by fluorescent microscopy of Hoechst-dye, stained DNA found in the cytoplasm of the 3T3 cells (3). No mycoplasma were found.

To detect MEP and MRP, cells were labeled with [^{35}S]-methionine as described before (1). In some experiments the [^{35}S]-methionine labeling medium was modified to include methionine at a concentration equal to 10% of the concentration present in DME. The [^{35}S]-labeled components in the medium were resolved by SDS polyacrylamide gel electrophoresis on 7.5-15% gradient gels as described previously (1). [^{35}S]-Methionine labeled bands were visualized using the method of Laskey and Mills (4), and the density of each band quantitated using a densitometer. Endoglycosidase H treatment of [^{35}S]-methionine labeled medium was done as described before (1).

MATERIALS

FGF was prepared from bovine brain (5). Other growth factors were gifts from Drs. S. Potter and R. W. Holley (EGF), and Dr. J. Patrick (NGF) (The Salk Institute). Monensin and nigericin were gifts from Dr. J. W. Chamberlin, Lilly Research Laboratories. Gramicidin D, chloroquine, and Con A were from Sigma. Amersham-Searle supplied [^{35}S]-methionine (600-1500 Ci/mmol).

RESULTS AND DISCUSSION

Effect of the Peptide Growth Factors on Extracellular MEP and MRP

Since the Balb/c 3T3 cells release much more MEP than MRP and the Swiss cells release much more MRP than MEP in response to the growth factors, we have used the Balb/c 3T3 cells to study MEP synthesis and release and the Swiss 3T3 cells to study MRP synthesis and release. The amount of extracellular MEP and MEP is selectively increased by EGF, FGF and serum (Table 1).

Concanavalin A and NGF also stimulate MEP and MRP release. The ability of these proteins to stimulate the appearance of extracellular MRP and MEP varies. In particular, EGF is a poor stimulator of MEP synthesis, and NGF and Con A are not as good as EGF and FGF in increasing extracellular MRP.

Effect of Monensin and Nigericin on the Amount of Extracellular MRP and MEP

The ionophore, monensin, exchanges Na^+ for H^+ in a model system (6) and possibly also in biological membranes (7,8). Nigericin mediates a similar

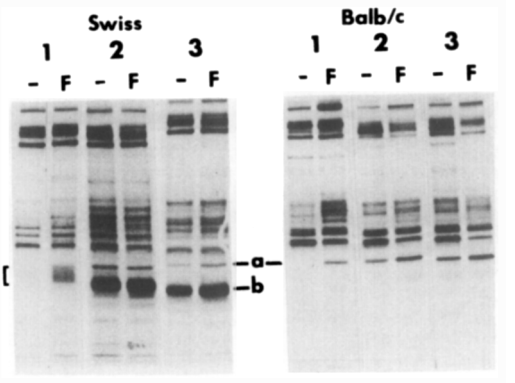

Fig. 1. Effect of monensin and nigericin on the amount of MRP and MEP released by Swiss and Balb/c 3T3 cells, in the presence and absence of FGF. 3T3 Cells were labeled with [^{35}S]-methionine as described previously (1), except that the methionine concentration in the medium used to label the Balb/c 3T3 cells was 10% of that normally found in DME. Cells were labeled with [^{35}S]-methionine for 4 hours in the presence (F) or absence (-) of FGF and with the additions of 1 μM monensin (2) or 0.1 μM nigericin (3). The control (1) contained 1% DMSO, which was present throughout. The cells were incubated with or without FGF for 20 hours (Swiss 3T3) or 16 hours (Balb/c 3T3) before labeling and also during the 4-hour labeling period. The ionophores were present only during the labeling period. The positions of MEP (a), MRP ([ ), and MRP' (b) are indicated.

exchange of K^+ for H^+ (8). Both monensin and nigericin increase the amount of extracellular MRP and MEP, in the presence and absence of FGF (Fig. 1). With the ionophores, the molecular weight of MRP - but not of MEP - drops from an average of 34,000 daltons to 30,000 daltons. Also, the MRP population released in the presence of monensin or nigericin, MRP', is narrower in molecular weight range than the original MRP. Like MRP, MRP' is glycosylated; that is, MRP' binds to lentil-lectin-Sepharose and can be eluted with α-methylmannoside. Removal of the glycosylated portion of MRP' with endoglycosidase H produces peptides of the same molecular weight as are produced from MRP. Neither monensin nor nigericin alter the apparent molecular weight of MEP, although MEP is also glycosylated; treatment with endoglycosidase H shifts the apparent molecular weight of MEP from 39,000 daltons to 33,000 daltons.

Effect of Agents That Increase Intralysosomal pH on the Amount of Extracellular MRP and MEP

Monensin increases cell pH in a neuroblastoma-glioma hybrid (7), possibly by exchanging Na^+ for H^+ (8). However, monensin and nigericin have also been shown to inhibit Na^+ transport in membrane vesicles isolated from SV40 transformed 3T3 cells (9). To determine whether the increased extracellular concentration of MEP and MRP' caused by monensin and nigericin could result

Table 1. Effect of mitogens and peptide growth factors on the amount of MEP and MRP released by 3T3 cells

	MEP (Balb/c 3T3)			MRP (Swiss 3T3)			
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 4	Expt 5	Expt 6
EGF	1.3	1.8	ND	12.2	ND	5.0	ND
FGF	7.6	5.1	7.9	15.2	4.6	5.4	6.8
NGF	ND	4.7	ND	ND	ND	ND	2.7
Con A	ND	ND	8.0	ND	ND	3.3	ND
Serum	ND	3.8	ND	ND	6.2	ND	ND

ND, not determined; Expt, experiment.

In a series of experiments, Balb/c and Swiss 3T3 cells were treated with various peptide or protein mitogens for 16 hours (experiments 2 - 6) or 20 hours (experiment 1). The mitogens were present at the following concentrations: 5 ng/ml EGF, 100 ng/ml FGF, 50 ng/ml NGF, 50 µg/ml Con A, and 10% serum. The cells were then labeled with [^{35}S]-methionine for 4 hours in the presence of the mitogens, as described previously (1). During the labeling period the mitogen concentrations remained the same as during the 16 - 20 hour preincubation but the serum concentration was reduced from 10% to 1%. The numbers shown, obtained from densitometer scans of autoradiograms, are the ratios of the areas under the MRP and MEP peaks obtained from the medium of 3T3 cells treated with the growth factors over the areas under the MRP and MEP peaks obtained from the medium of 3T3 cells treated with the solution in which the growth factors were dissolved (bovine serum albumin, 0.005%, final concentration).

from the ability of these ionophores to transport H^+, we tested the effect of agents known to alter intracellular or intralysosomal pH (Table 2). All compounds expected to increase intralysosomal pH also increase the amount of extracellular MRP' and MEP. Compounds not expected to alter intralysosomal pH - valinomycin, ionophore A23187, gramicidin D - do not cause an increase in extracellular MRP; but treatment with gramicidin D does cause a shift in the average molecular weight of MRP towards that of MRP'. Extracellular MRP' concentrations are also increased by raising the extracellular pH, another method of increasing the intracellular pH (10).

In 3T3 cells, the concentration range in which monensin stimulates the appearance of extracellular MRP' and MEP (0.2 µM - 1 µM) is 10-times higher than the concentration range in which monensin inhibits the release of collagen and fibronectin from human fibroblasts (11). We find that, in 3T3 cells, general inhibition of glycoprotein secretion occurs at 10 µM monensin,

Table 2. Effect of agents that change intracellular ion concentrations on the appearance of extracellular MRP and MEP

Agent	MRP		MEP
	Stimulated	Decreased Molecular Weight	
Valinomycin	-	-	ND
Gramicidin D	-	±	ND
A23187	-	-	ND
Nigericin	+	+	+
Monensin	+	+	+
NH_4Cl	+	+	+
Methylamine	+	+	ND
Chloroquine	+	+	+
Extracellular pH 8.0 - 8.5	+	+	ND

ND, not determined.

Labeling with [^{35}S]-methionine was done as described in Table 1, although there was no preincubation with mitogen. The cells were labeled for 4 hours and the agent was added with the labeling medium. Each agent was tested over a range of concentrations: valinomycin (0.001 - 1 μM), gramicidin D (0.01 - 10 μg/ml), A23187 (0.001 - 1 μM), nigericin (0.001 - 10 μM), monensin (0.001 - 10 μM), NH_4Cl (0.01 - 20 mM), methylamine (10 mM), and chloroquine (1 - 20 μM). In most cases, the highest concentration was inhibitory. Approximate values for the half-maximal effective concentrations were: monensin (0.1 μM), nigericin (0.04 μM), and ammonium chloride (5 - 10 mM). Chloroquine was effective in the range of 5 to 20 μM. Consistent positive (+) and negative (-) effects are indicated. An inconsistent and usually incomplete effect is also indicated (±).

a concentration about 10-times higher than that which increases the extracellular concentrations of MRP' and MEP.

When MRP or MRP' is digested with endoglycosidase H, identical smaller molecular weight forms are produced. Therefore, the difference in apparent molecular weight between MRP and MRP' is probably due to different degrees of glycosylation. To produce MRP' from MRP, the pH-altering agents could inhibit glycosylation or stimulate deglycosylation. The observation that gramicidin D causes a shift in the apparent molecular weight of MRP but does not increase the amount of extracellular MRP' suggests that the shift in molecular weight and the increase in extracellular concentration of MRP are two independent

events. That the alteration in molecular weight is not necessary for the increased extracellular appearance of MRP is also suggested by the observation that the molecular weight of MEP, which we have found is also a glycoprotein, is not altered when the amount of extracellular MEP is increased.

By immunofluorescence, MEP was found in cytoplasmic granules (2). These could be secretory vesicles or lysosomes. Ammonium chloride and chloroquine increase the amounts of lysosomal enzymes secreted by mouse macrophages (12) and human fibroblasts (13). Since the amount of MRP is regulated in a similar manner to MEP, it is possible that MEP and MRP share the same intracellular compartment.

The extracellular concentrations of some lysosomal enzymes are increased by inhibiting their reuptake by the cells. For example, ammonium chloride and chloroquine inhibit receptor-mediated uptake of α-L-iduronidase by human fibroblasts (14) and [^{125}I]-mannose-BSA by macrophages (15). Also, methylamine inhibits the receptor mediated uptake of α_2-macroglobulin by NRK cells (16) and human skin fibroblasts (17). However, it is unlikely that the pH altering agents increase extracellular MRP' by inhibiting its reuptake because we have been unable to show that Swiss 3T3 cells deplete MRP from the extracellular medium either by taking it up or by degrading it (1).

Ammonium chloride, chloroquine, and methylamine inhibit protein degradation that occurs in the lysosomes (18,19); they probably do this by increasing intralysosomal pH. Monensin and nigericin should move H^+ in opposite directions across the plasma membrane, since K^+ is more concentrated intracellularly and Na^+ more concentrated extracellularly; but, because of the high intralysosomal H^+ concentration, both ionophores should move H^+ out of the lysosomes - raising the intralysosomal pH and thereby inhibiting protein degradation. Nigericin has been shown to inhibit protein degradation in rat liver phagolysosomes (20).

If extracellular MRP and MEP concentrations are regulated by intracellular protein degradation, then our data suggests that at least 50% of these proteins are normally degraded before being released - probably as small peptides. Although MRP and MEP could be lysosomal proteins, they could also be located in secretory vesicles which occasionally collide and fuse with the lysosomes; for example, there is evidence that a fraction of the populations of the secreted proteins, prolactin (21), parathormone (22), and collagen (23) are degraded intracellularly before secretion. If, as in the case of these secreted proteins, MRP and MEP have extracellular functions, then intracellular degradation may serve to regulate extracellular levels of the proteins or to remove defective molecules before they are secreted (24). Alternatively, MEP and MRP might have intracellular functions; in this event, both degradation

and secretion would serve the purpose of regulating intracellular levels of these proteins.

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