Regulation of mammary hormone receptor metabolism by a retroviral envelope protein

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ABSTRACT
In a previous study, the envelope protein (gp52) of the mouse mammary tumour virus (MMTV) was shown to facilitate mammary gland differentiation by increasing prolactin (PRL) receptors via increased receptor synthesis and via the redistribution of existing receptors from an internal pool. In this study, receptors for other hormones known to affect mammary gland metabolism were investigated. Epidermal growth factor (EGF) stimulates mammary epithelial growth and inhibits differentiation; its receptor is rapidly and dramatically down-regulated by gp52. This is accomplished by its internalization and by decreasing its half-life from 27 h to 2.4 h. Surprisingly, it also increased EGF receptor synthesis, although this effect was not great enough to overcome receptor down-regulation. In contrast, gp52 did not affect the distribution, half-life or synthesis of the insulin receptor. These results demonstrate that MMTV can enhance mammary differentiation by coordinately regulating several hormone receptors: specifically, it can increase the number of receptors for PRL, a differentiative hormone, while decreasing the number of receptors for EGF, a growth/anti-differentiative hormone.

Journal of Molecular Endocrinology (1998) 21, 161–168

INTRODUCTION
The mouse mammary tumour virus (MMTV) is a retrovirus that is propagated from mother to pups via the milk (Nandi & McGrath 1973). Although it is oncogenic, the MMTV genome does not contain any oncogenes, and tumours only occur after a long latency period (van Leeuwen & Nusse 1995). As such, mammary growth is a late indirect effect of the MMTV; instead, it is mammary differentiation and lactation that is critical for the propagation of the virus. To facilitate its own reproduction, the MMTV envelope protein, gp52, binds to the mammary cell surface and enhances the epithelial sensitivity to prolactin (PRL), a differentiative hormone (Bolander 1994a). This change in responsiveness is primarily accomplished by increasing PRL receptors via increased receptor synthesis and recruitment of PRL receptors from an intracellular pool (Bolander et al. 1997).

The question arises as to whether this is an isolated effect of the virus or whether it is part of a concerted programme to enhance mammary differentiation. To explore this question, the effects of gp52 on the metabolism of other hormone receptors were examined. In particular, two hormone receptors with totally different functions, as compared with the differentiative role of PRL receptors, were studied: the epidermal growth factor (EGF) receptor, which mediates growth, and the insulin receptor, which is responsible for the maintenance of general metabolism.

MATERIALS AND METHODS
Materials
Mouse EGF (lot 908374) was purchased from Collaborative Biomedical Products (Bedford, MA, USA), and crystalline porcine insulin (lot 092WW6) was a gift from Eli Lilly Company (Indianapolis, IN, USA). Cortisol, tri-iodothyronine (T3), Hepes, cycloheximide, lactoperoxidase, BSA, normal mouse serum, goat anti-mouse immunoglobulin G and Dulbecco’s modified Eagle’s medium without cysteine or methionine were obtained from Sigma Chemical Company (St Louis, MO, USA). Medium 199 with Earle’s salts was from Grand Island Biological Company (Grand Island, NY, USA) and collagenase type I (179 U/mg) was purchased from Worthington Biochemicals (Freehold, NJ, USA). Monoclonal antibodies to the mouse EGF receptor (lot 2) and insulin receptor (lot 2) were obtained from Transduction...
Laboratories (Lexington, KY, USA), and reagents for SDS-PAGE were purchased from Bio-Rad (Richmond, CA, USA). A mixture of [35S]cysteine and [35S]methionine (Tran35S-Label; >1000 µCi/mm) was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA) and Na125I (carrier-free) was from New England Nuclear (Boston, MA, USA).

gp52, the MMTV envelope protein, was prepared in my laboratory by the method of Marcus et al. (1979); milk from C3H/HeN MMTV+ mice was used as the source of the virus. No contamination with other proteins could be detected on overloaded gels (Bolander et al. 1997). Polyclonal antibodies to gp52 were also produced in my laboratory as described in Bolander & Blackstone (1990).

Animals and tissue preparation
Virgin mice (C3H/HeN MMTV+ and MMTV−) were obtained from the Frederick Cancer Research Facility (Frederick, MD, USA). The mice were killed by cervical dislocation. After the lymph nodes had been removed from the fourth pair of glands, explants were prepared under sterile conditions, as previously described (Juergens et al. 1965). Explants were cultured on siliconized lens paper in Medium 199 containing 20 mM Hepes (pH 7·6) and combinations of the following reagents, as required by the individual experiment: cycloheximide (10 µg/ml), insulin (1 µg/ml), cortisol (1 µg/ml), T3 (65 pg/ml) and/or gp52 (1 µg/ml). The concentration of gp52 was chosen so as to saturate the gp52 receptor (Bolander & Blackstone 1991). The tissue was incubated under air at 37 °C, and the medium was changed daily.

Cell isolation and membrane fractionation
EGF and insulin receptors were measured on an epithelial cell-enriched fraction isolated from mammary explants, as previously described (Vonderhaar et al. 1973). Briefly, the tissue was finely minced and digested with collagenase (1·5 mg/ml of Medium 199 containing 20 mM Hepes (pH 7·6) and 4% (w/v) BSA) at 37 °C. During this incubation, the tissue fragments were pipetted through successively smaller bore pipettes. After 30 min, the cells were centrifuged at 800 g for 30 s and washed three times in Medium 199 containing 20 mM Hepes (pH 7·6) and 2% BSA. The resulting preparation contained small pieces of ducts and end buds, rather than individual epithelial cells. In addition to minimizing damage to the epithelium, this technique easily separates the epithelial aggregates from isolated fibroblasts which sediment more slowly.

When EGF and insulin binding in membrane fractions was to be assessed, the epithelial cells were lysed and fractionated using the two-phase system of Brunette & Till (1971). This technique separates the plasma membrane from other fractions, referred to here as microsomes. Because receptor redistribution can occur quickly (Bolander 1994b), a 30 min time point was chosen for the determination of both EGF and insulin receptors; the distribution of EGF receptors was also measured after 3 days to determine whether any of the observed changes persisted. However, because the insulin receptor can be regulated by both insulin and glucocorticoids (Knutson et al. 1982), these hormones were omitted from the medium used for insulin receptor studies. Since the tissue remained viable for at least several hours without insulin and cortisol, the 30 min time point and the receptor half-life studies were not affected by this omission. However, the 3 day time point was not possible in the insulin receptor experiments.

EGF and insulin receptor assay
EGF and insulin were iodinated by a modification (Bolander & Fellows 1975) of the lactoperoxidase method of Miyachi et al. (1972). The resulting 125I-labelled hormone was used in binding studies, as previously described (Bolander 1984). Binding was saturable, and 80–90% of the tracer could be dissociated upon dilution, after which the receptor could be recovered and shown to rebind approximately the same amount of 125I-labelled hormone, suggesting that the receptor was not damaged during the 2 h incubation. The radioactivity in the supernatant could be completely precipitated by trichloroacetic acid after 2 h, suggesting that it too was not degraded.

For the receptor half-lives, mammary explants were cultured in Medium 199 with insulin, cortisol and cycloheximide (10 µg/ml); all additives were co-mixed simultaneously. At various time points, cells were isolated and total receptors were measured on the plasma membrane as described above.

Protein was determined by the method of Lowry et al. (1951), and binding data were analysed by the method of Scatchard (1949). Because of the low epithelial content of mouse mammary glands, five to six animals were required to generate enough cells to construct a single Scatchard plot.

Synthesis of EGF and insulin receptors
Receptor synthesis was determined by incubating explants in 2 ml Dulbecco’s modified Eagle’s
medium deficient in cysteine and methionine but containing 20 mM Hepes (pH 7.6). The explants were cultured with or without gp52; after 4 h, 100 µCi of a mixture of \(^{35}\)S)cysteine and \(^{35}\)S)methionine was added and the tissue cultured for 2 h more. This time period was chosen because preliminary experiments showed that there was no effect of gp52 on the synthesis of either the insulin or EGF receptors before 4 h. The explants were then homogenized and the receptors immunoprecipitated as previously described (Ono & Oka 1980). The precipitate was dissolved in PBS containing 4 M deionized urea and 2 M β-mercaptoethanol and electrophoresed on SDS–polyacrylamide gels by the method of Weber & Osborn (1969). The major radioactive bands corresponded to the known molecular masses for the EGF receptor (170 kDa) and the α and β chains of the insulin receptor (95 and 135 kDa respectively). These bands were excised, eluted and counted for radioactivity.

RESULTS

EGF receptor levels in mammary glands from pregnant mice fluctuate markedly, first rising in early pregnancy and then declining later; these low levels then persist during lactation (Edery et al. 1985). Virgin mice were chosen for this study, because EGF receptor levels in glands from these animals are stable, easily measured and uninfluenced by the high concentrations of hormones associated with pregnancy and lactation.

All Scatchard plots of the EGF receptor data were linear (Fig. 1) and the affinity constants did not differ significantly among experimental groups using ANOVA (69 ± 12 pM). In mammary epithelial cells from MMTV\(^{-}\) mice, nearly 90% of the EGF receptors were located on the cell surface (Fig. 2). Within 30 min of exposure to gp52, these plasma membrane receptors decreased by one-third. At the same time, microsomal EGF receptors increased by a similar amount, suggesting that the receptors had been internalized. However, by 3 days the microsomal EGF receptors had returned to baseline values, while the plasma membrane receptors continued to fall. This suggests that the EGF receptors were not being recycled to the cell surface but were destroyed. This result is consistent with reports that the EGF receptor does not recycle in many tissues, leading to a prolonged period of down-regulation (Das 1982).

Since EGF has similar effects on its own receptor (Haigler et al. 1978, Schlessinger et al. 1978) and since gp52 was purified from viruses obtained from milk, which also contains EGF (Gresik et al. 1984), the gp52 used in this study was evaluated for possible contamination with EGF. If the effects of gp52 were due to contaminating EGF, they should be blocked by EGF receptor antibodies. Table 1

![Figure 1](image1)

FIGURE 1. Representative Scatchard plot of EGF receptors on the plasma membrane of uncultured epithelial cells from MMTV\(^{-}\) mice.

![Figure 2](image2)

FIGURE 2. EGF receptor redistribution in mammary epithelium from MMTV\(^{-}\) mice. Mammary explants were incubated either with (○, ★) or without (□, ■) gp52, and EGF receptors were measured in plasma membranes (★, ■) or microsomes (○, □) at the indicated times. Values are means ± s.e.m. (n=4).
shows that, although these antibodies can inhibit EGF-induced receptor down-regulation, they cannot prevent the effects of gp52. However, antibodies specific to gp52 can block the actions of gp52. In addition, gp52 is unable to displace 125I-labelled EGF from EGF receptors (Table 2). Therefore the effect of gp52 on EGF receptors is not due to EGF contamination.

The redistribution of EGF receptors induced by gp52 is further supported by studies on the half-life of the EGF receptor (Fig. 3). In unstimulated epithelium, receptor levels declined logarithmically in the presence of cycloheximide; the estimated half-life was 27 h, a value similar to that reported by Edery et al. (1989) in this same tissue. However, in the presence of gp52, the receptor half-life fell dramatically to only 2·4 h (P<0·01).

Surprisingly, gp52 stimulated EGF receptor synthesis 5- to 6-fold by 6 h (Fig. 4). This seeming paradox has also been observed during EGF-induced down-regulation: although EGF profoundly down-regulates its own receptor, it simultaneously elevates EGF receptor mRNA 5-fold (Clark et al. 1985, Earp et al. 1986, Bjorge & Kudlow 1987). It has been postulated that this effect primes the cell so that it can rapidly replenish its EGF receptor numbers after EGF has been removed.

Unlike the EGF receptor plots, the Scatchard plots for the insulin receptor data were curvilinear, confirming the reports of others (Inagaki & Kohmoto 1982, Bolander 1984). Because of the questionable physiological relevance of the low-affinity sites, only the high-affinity receptors were examined. The affinity constants for these latter sites did not differ significantly among the experimental groups (360±30 pM); therefore, only receptor numbers are given. Like the EGF receptors, insulin receptors were predominantly located in the plasmalemma (Fig. 5). However, in contrast with the EGF receptors, the insulin receptors did not undergo a redistribution in response to gp52. Furthermore, there was no significant change in insulin receptor half-life: 7·5 vs 8·4 h for epithelium cultured with and without gp52 respectively (Fig. 6). Finally, gp52 had no effect on insulin receptor synthesis (Fig. 4).

DISCUSSION

As noted in the Introduction, MMTV propagation is dependent upon lactation, since this is the route by which the pups become infected. Furthermore,
MMTV production begins in late pregnancy, when morphological development is nearing completion and milk production is imminent (Muñoz & Bolander 1989). At this time, the MMTV is shed into both the milk and the bloodstream (Nandi & McGrath 1973), from which the envelope protein, gp52, can activate its receptor. Previous studies from this laboratory have shown that this interaction can facilitate biochemical differentiation in the mammary gland by elevating PRL receptors (Bolander et al. 1997). The data from this work demonstrate that gp52 can also decrease EGF receptors.

How can a change in EGF receptors affect MMTV propagation? In many tissues, growth factors and differentiative factors oppose each other. For example, in mouse mammary explants, EGF inhibits casein synthesis, a marker for biochemical differentiation (Taketani & Oka 1983). In addition, EGF receptors in mammary epithelium decrease during late pregnancy and lactation (Edery et al. 1985), when PRL receptor levels are rising and biochemical differentiation is commencing (Holcomb et al. 1976). However, subsequent studies have complicated the interpretation of these earlier works, suggesting that the inhibitory effects in vitro may have been dependent upon the presence of other hormones (Vonderhaar & Nakhasi 1986, Sankaran & Topper 1987). Furthermore, EGF is known to enhance morphological differentiation in mouse glands, i.e. lobuloalveolar development (Tonelli & Sorof 1980, Vonderhaar 1987). Finally, sialoadenectomized mice, which cannot synthesize normal amounts of EGF (Okamoto & Oka 1984), and homozygous waved-2 mutant mice, which have mutant EGF receptors and cannot respond to EGF (Fowler et al. 1995), have impaired lactation. The general consensus is that EGF is necessary for mammary epithelial proliferation, which affects the magnitude of lactation, but that EGF is detrimental to actual milk production (Forsyth 1989, Oka et al. 1991). As noted above, blood MMTV levels do not rise until late pregnancy, when it could block the late anti-differentiative effects of EGF without affecting the earlier effects of EGF on mammary growth. As such, MMTV enhances mammary differentiation by increasing the receptors for PRL, a differentiative hormone, while decreasing those for EGF at a time when its anti-differentiative effects would be manifest.

Other viral proteins have also targeted EGF receptor metabolism. In contrast with the MMTV, the human papillomavirus is dependent upon cellular replication for its propagation. To this end, the E5 protein of this virus binds to and inhibits
the vacuolar H⁺-ATPase, which is required for the proper acidification of endosomes (Straight et al. 1995). As a result, the internalized EGF receptor escapes destruction and is recycled to the cell surface where it mediates cellular proliferation.

The function of insulin is often described non-specifically as ‘maintenance of viability’; as such, one might not expect it to have a specific role in either growth or differentiation. However, like EGF, the function of insulin in the mammary gland is complicated: it is specifically required for the induction of milk proteins, and insulin-like growth factors cannot substitute for it (Bolander et al. 1981, Nicholas & Topper 1983). In addition, the insulin receptor increases during pregnancy and remains increased during lactation (Inagaki & Kohmoto 1982), suggesting an important role in differentiation. However, its receptor does not appear to be a target of the MMTV; receptor synthesis, distribution and half-life are all unaffected by gp52. Apparently, the effects of insulin on differentiation are not sufficiently specific or limiting to favour the evolutionary acquisition of regulatory controls over these receptors by the MMTV.

Many viruses use their cellular receptors not only to identify target tissues and then gain entry, but also to activate physiological functions that facilitate viral propagation. Most viruses require ongoing cellular replication for their propagation and choose their receptors accordingly, e.g. the vaccinia virus binds to and activates the EGF receptor (King et al. 1986), while the papillomavirus binds to and activates the platelet-derived growth factor receptor (Kulke & DiMaio 1991). In contrast, the propagation of the MMTV is dependent upon the differentiation of the mammary epithelium, and it employs a rather sophisticated strategy to aid its own cause. Although its envelope protein does not directly bind to either the PRL or EGF receptors, activation of the binding protein for gp52 does affect these receptors: it increases the number of PRL receptors and decreases the number of EGF receptors, thereby enhancing differentiation by facilitating the action of PRL, a lactogenic hormone, and impairing the effects of EGF, a growth/anti-differentiation hormone.

ACKNOWLEDGEMENTS

This work was supported in part by Public Health Service grant CA 42009 from the National Cancer Institute. The technical assistance of William McAmis is gratefully appreciated.

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Journal of Molecular Endocrinology (1998) 21, 161–168

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REVISED MANUSCRIPT RECEIVED 13 January 1998