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Lovastatin suppresses erythropoietin receptor surface expression through dual inhibition of glycosylation and geranylgeranylation

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ABSTRACT

Erythropoietin (Epo) is a cytokine that is required for the survival of erythroid progenitors through interaction with its receptor on the surface of these cells. Recent studies showed that erythropoietin receptor (EpoR) is expressed on many cancer cells. The factors that govern EpoR expression on the cell surface are poorly understood. Using both biotinlyation and radiolabeled Epo binding experiments, we show here that Epo starvation of the Epo-dependent erythroleukemia cell line, ASE2, leads to a time-dependent increase in both forms of EpoR, the maturing 64 kDa and the mature 66 kDa proteins. Mevalonate depletion inhibits the formation of the highly glycosylated mature form of EpoR without affecting the other form. Treatment of cells with lovastatin, a selective inhibitor of the rate-limiting enzyme in the mevalonate pathway leads to inhibition of cell surface EpoR that is induced by Epo starvation. The effect of lovastatin appears to be the consequence of inhibition of two processes, glycosylation and geranylgeranylation. Adding back geranylgeranyl pyrophosphate to lovastatin-treated cells completely prevents the lovastatin effect on EpoR expression. Dolichol, the sugar carrier in N-linked glycosylation that is derived from the mevalonate pathway, partially reverses lovastatin's effect. The glycosylation inhibitor tunicamycin also partially suppresses EpoR surface expression. Inhibiting protein geranylgeranylation mimics the effect of lovastatin and inhibits EpoR surface expression in a concentration-dependent manner. Finally, lovastatin inhibits Epo's stimulatory effects on cell proliferation. These results indicate that mevalonate derivatives are required for normal EpoR expression on the cell surface through two pathways, glycosylation and geranylgeranylation.

1. Introduction

Erythropoietin (Epo) is a member of the class I cytokine family and is a major regulator of red blood cell production [1,2]. Binding of Epo to its receptor (EpoR) on erythroid progenitors is required for the survival, proliferation, and ultimate differentiation of the cells [3]. These effects are initiated by Epo-induced activation and phosphorylation of Jak2, a kinase that is constitutively associated with EpoR [4,5]. Upon activation, Jak2 phosphorylates EpoR at several tyrosines that then act as recruitment sites for many SH2 domain-containing intermediates like Stat5 [6–9] and Grb2 [10–12]. These proteins are

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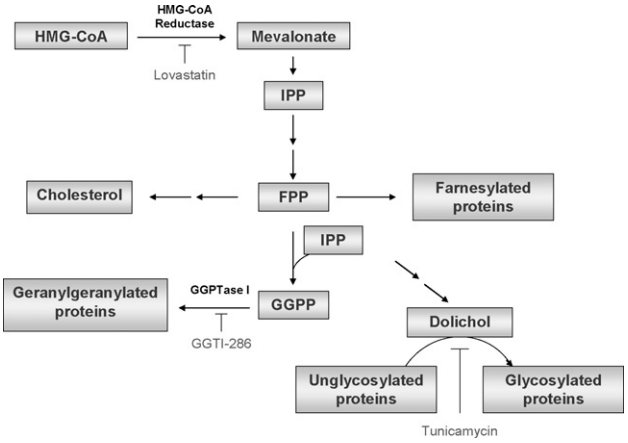

Fig. 1 – The mevalonate biosynthetic pathway.

consequently activated to transduce the Epo signal to downstream effectors, such as the Ras/Raf/Erk pathway in the case of Grb2 [10,13,14]. Recent studies have shown that functional EpoR is expressed on many non-hematopoietic cancer cells and that signaling through this receptor contributes to the proliferation and migration of these cells [15,16].

The EpoR is synthesized as a 62 kDa precursor that is quickly modified by glycosylation to become a 64 kDa protein. The mature EpoR exhibits a 66 kDa molecular mass and a complex Golgi-processed endoglycosidase H-resistant glycosylation pattern [17,18]. In normal erythroid cells, the level of EpoR expression on the surface is very low [19]. The cell surface expression of EpoR appears to be tightly regulated by mechanisms that are poorly understood [20].

The mevalonate biosynthetic pathway (Fig. 1) provides intermediates that are crucial for cell survival and function [21]. Cholesterol is one major example. Hypocholesterolemic agents, such as lovastatin, act through inhibiting the rate-limiting enzyme in the mevalonate pathway, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. However, besides cholesterol, other products of this pathway are of vital importance to the functions of the cell. For example the isoprenoids, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are required for post-translational modification of proteins such as Ras and Rho through farnesylation and geranylgeranylation, respectively [22–26]. These modifications allow the small GTPases to localize to their sites of action in cellular membranes [27]. Depletion of the isoprenoid units results in loss of proper localization and hence functioning of these proteins [28,29].

Dolichol is another product of the mevalonate pathway. This large isoprenoid compound acts as a carbohydrate donor during N-linked glycosylation of membrane-targeted proteins [30]. Dolichol is also thought to play a role in the processing of the oligosaccharides in the Golgi apparatus [31,32]. In addition to EpoR, a large number of cell surface receptors are glycosylated. Some of these receptors, such as IGF-1 and insulin receptor, require glycosylation for correct processing and activity [33–35]. The role of glycosylation in EpoR processing and cell surface expression is not completely understood.

We have previously shown that protein geranylgeranylation is required for proper EpoR signal transduction [36]. Depletion of GGPP or inhibition of geranylgeranyl transferase, the enzyme that modifies small GTPases such as Rho and Rap, results in inhibition of Epo-induced phosphorylation of Jak2, Stat5, and Erk. However, the exact mechanism for this inhibition of Epo signaling is not clear. Here we extend these findings to show that mevalonate depletion leads to inhibition of maturation of EpoR and its expression on the cell surface. This effect appears to be the sum of inhibition of two processes: protein glycosylation and geranylgeranylation.

2. Materials and methods

2.1. Antibodies and reagents

EpoR, Erk, and phospho-Erk antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IL-3R (β), Jak2, Stat5, and phospho-Stat5 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The pan-Ras antibody was kindly provided by Dr. Setsuo Hirohashi (National Cancer Center, Tokyo). Lovastatin, mavalonolactone, FPP, GGPP, tunicamycin and MTT were obtained from Sigma–Aldrich (St. Louis, MO). Lovastatin and mevalonolactone were activated by dissolving in 0.1N NaOH to generate the respective open acid forms and the pH adjusted to 7.4 with 0.1N HCl. GGTI-286 was obtained from EMD Biosciences (San Diego, CA). Dolichol was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]glucosamine was purchased from Moravek Biochemicals (Brea, CA). [^{125}I]Epo was purchased from GE Healthcare.

2.2. Cell culture

The Epo-dependent leukemia cell line, ASE2 [37], was a generous gift from Chugai Pharmaceutical Company (Tokyo, Japan). Cells were grown in Iscove's Modified Dulbecco's Medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum, penicillin, streptomycin, and 10 U/ml erythropoietin. Cell cultures were maintained at 37 °C, 5% CO_2, and saturating humidity. Cells (32 Da) were cultured as described previously [36].

2.3. Cell lysing and Western blotting

ASE2 cells were lysed in RIPA lysing buffer (1% sodium deoxycholate, 1% Triton, 0.1% SDS, 1 mM PMSF, 50 mM Tris, 150 mM NaCl, protease inhibitor cocktail [Sigma]). The clarified cell lysates were resolved by 7, 10, or 15% SDS-PAGE and subsequent Western blotting. For the immunoprecipitation experiments NP-40 lysing buffer (0.5% NP-40, 10% glycerol, 50 mM Tris, 0.1 mM EDTA, 150 mM NaCl, protease inhibitor cocktail) was used instead of RIPA.

2.4. Analysis of receptor surface expression using biotinlyation

The cell surface protein biotinylation and purification kit (Cat # 89881, Pierce, Rockford, IL) was used for the isolation of surface proteins according to the manufacturer's instructions. Briefly, after each treatment cells were biotinylated using a cell-

impermeable biotinylating agent at 4 °C for 30 min. Cells were then lysed and cell lysates were run on Streptavidin column to collect the biotinylated fractions that correspond to cell surface proteins. Subsequently, the collected fractions were eluted using SDS and run on SDS-PAGE. Western blot analyses were performed using EpoR or IL-3R antibodies.

2.5. Radiolabled Epo binding studies

Saturation binding studies were performed as described previously. Briefly, ASE2 cells were treated as indicated. Subsequently 2×10^6 cells were incubated with 3 nM [^{125}I]Epo (specific activity 2500 Ci/mmol) in 100 μl binding buffer (PBS, 0.1% BSA) at 4 °C for 3 h. Cells were then washed three times with ice-cold PBS. The radioactivity in the final cell pellet was measured using gamma counter. Nonspecific binding was calculated in the presence of 500× cold Epo and subtracted from total binding. CPMs were converted to DPMs based on counting efficiency and surface Epo binding sites were derived from the following formula:

$$\text{total binding sites/cell} = \frac{\text{CPM}}{\text{counting efficiency (CPM/DPM)} \times 2.22 \times 10^{12}\ \text{DPM/Ci} \times \text{specific activity (2500 Ci/mmol)}} \times \frac{10^{-3}\ \text{mol/mmol} \times \text{Avogadro's constant}\ (6.022 \times 10^{23}\ \text{site/mol})}{\text{number of cells}}.$$

2.6. Determination of EpoR glycosylation

EpoR glycosylation studies were performed according to the method of Carlberg et al. [35]. Briefly, cells were treated with the various agents as described in subsequent text. During the last 4 h of each treatment, cells were labeled with 20 μCi/ml D-[6-^{3}H]glucosamine (1 mCi/ml, 39.9 Ci/mmol) followed by lysing in NP-40 lysing buffer. Protein concentrations of the cell lysates were determined and equal amounts were incubated with EpoR antibody for 24 h at 4 °C. Protein A-Sepharose was then added for 4 h. Beads were collected by centrifugation and washed several times with ice-cold PBS. Radioactivity was measured in the immunoprecipitates using scintillation counting.

2.7. Determination of total N-linked glycosylation

Total glycosylation was measured as described previously [36]. Briefly, cell cultures were treated as indicated. During the last 4 h of each treatment, cells were labeled with D-6-[^{3}H]glucosamine (10 μCi/ml, 39.9 Ci/mmol). Thereafter cells were rinsed three times with PBS and treated with cold trichloroacetic acid (TCA). The precipitate was then washed with 10% TCA and solubilized in tissue solubilizer (TS-2) overnight at room temperature. Radioactivity was determined by scintillation counting and normalized to protein content.

2.8. Cell survival studies

MTT studies were performed as suggested by Sigma. MTT was added for the last 4 h of each treatment.

3. Results

3.1. Lovastatin inhibits the formation of the fully glycosylated mature EpoR

We have previously shown that lovastatin inhibits signal transduction through EpoR [36]. In order to understand the mechanism that underlies this effect, EpoR processing was evaluated in both basal and Epo-starved states in ASE2 cells. Previous studies have shown that EpoR runs as two major bands, 64 and 66 kDa, on SDS-PAGE [17,18]. The former represents the maturing EpoR while the latter corresponds to the fully mature EpoR. The difference in molecular weights is due to difference in glycosylation states of the two forms.

Interestingly, starving cells of Epo leads to a time-dependent increase in both the maturing 64 kDa and the mature, fully glycosylated, 66 kDa forms of EpoR (Fig. 2A). Treating the cells with lovastatin suppresses the formation of the mature, fully glycosylated, 66 kDa form while not affecting the maturing 64 kDa form. Notably, total levels of EpoR decrease with lovastatin treatment since lovastatin does not increase the abundance of the 64 kDa form. Since Jak2 was shown to be required for maturation of EpoR [38], we studied the levels of Jak2 under these circumstances. As Fig. 2A shows, Jak2 levels are not affected by Epo starvation or by lovastatin treatment.

EpoR levels were also measured upon stimulation of ASE2 with Epo after 20-h starvation in the absence or presence of lovastatin (Fig. 2B). In control cells, Epo stimulation leads to a time-dependent decrease in the level of the mature 66 kDa form of EpoR. This effect is expected and is presumed to be a result of EpoR degradation [20,39]. Of interest is that lovastatin treatment decreases the starvation-induced expression of the mature 66 kDa form of EpoR at 0 min and prevents the change in EpoR levels upon Epo stimulation (Fig. 2B). Consistent with our prior observation, lovastatin treatment decreases the phosphorylation levels of Stat5 and Erk without affecting the total level of the two proteins.

3.2. Lovastatin inhibits surface expression of EpoR

In order to gain further insight of the effect of mevalonate depletion on EpoR processing, EpoR expression on the cell surface was measured using biotinylation experiments. Cells were incubated with or without lovastatin for 20 h in the presence of Epo. Subsequently, cells were starved or not (basal condition) from Epo for different periods of time. Surface proteins were then isolated using biotinylation methods as described above. Western blot analysis was then carried out using EpoR antibody. As shown in Fig. 3, Epo-starvation increases EpoR cell surface expression in a time-dependent manner. This result is in agreement with findings by others.

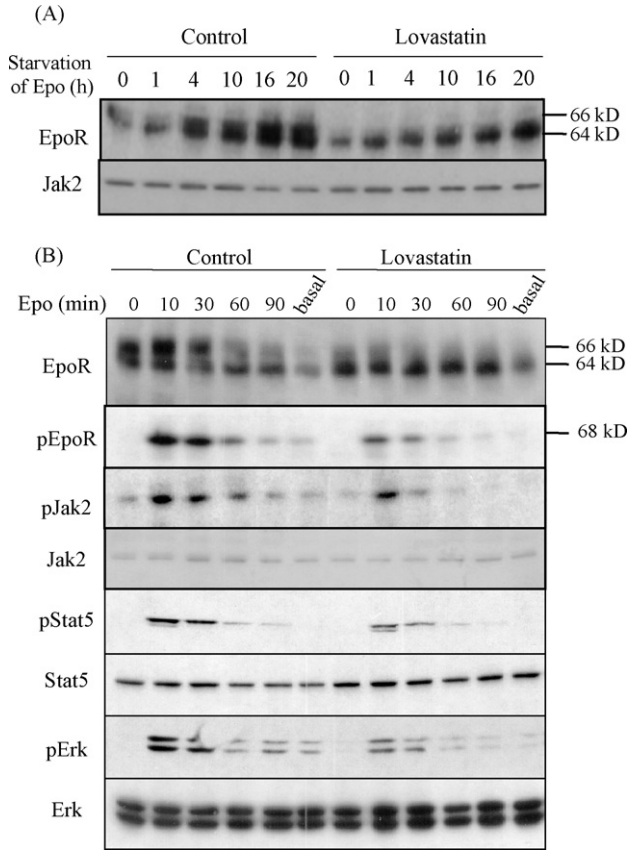

Fig. 2 – Mevalonate depletion inhibits the formation of the fully glycosylated mature form of EpoR upon Epo starvation. (A) ASE2 cells were treated or not (control) for 20 h with 20 μM lovastatin in the presence of Epo and then starved of Epo in the presence of lovastatin for the indicated time points and lysed. Proteins were detected by Western blot analysis using 7% SDS-PAGE. (B) ASE2 cells were treated for 20 h with lovastatin in the presence of Epo then starved of Epo for 20 h. Cells were then stimulated with 10 U/ml Epo for the indicated time points and lysed. Proteins were detected by Western blot analysis using 10% SDS-PAGE and the indicated antibodies. Shown are representative blots for triplicate experiments.

Mevalonate depletion by lovastatin treatment inhibits the upregulation of EpoR on the cell surface. This is consistent with the finding that in cell lysates, there is a diminution of the levels of the mature 66 kDa form of EpoR (Figs. 2A and 3, EpoR cell lysate).

To further confirm our findings and validate the results of the biotinylation experiment we performed radiolabeld Epo binding studies to accurately measure the number of Epo binding sites on the cell surface. Cells were starved for different time points in the presence or absence of lovastatin. After the starvation period cells were incubated with saturating concentrations of [^{125}I]Epo for 3 h at 4 °C to prevent internalization or recycling of the receptor. Subsequently cells were washed and radioactivity in the cell pellet was measured using a gamma counter. Consistent with findings from biotinylation experiments, starvation of Epo increases the number of binding sites in a time-dependent manner. For example, 4 h starvation increased the number of Epo binding sites from 1802 to 4273 and 24 h starvation quadrupled it to 8216 binding site (Fig. 3C). Interestingly lovastatin treatment inhibited this increase in Epo binding sites upon Epo starvation and slightly decreased the number of binding sites under basal conditions (Fig. 3C). These results support our previous findings (Fig. 3) and confirm the validity of the biotinylation experiment which we continued to use for the coming studies.

3.3. *GGPP prevents lovastatin's effects*

Since lovastatin inhibits the rate-limiting step in the synthesis of isoprenoids (Fig. 1), it depletes the cells of all the intermediates in the mevalonate pathway. In order to clarify the specific product(s) that is/are responsible for lovastatin's effects, add-back experiments were performed. Cells were cultured in the absence or presence of lovastatin. Lovastatin-treated cultures were additionally supplemented with mevalonate, FPP, FPP + IPP, or GGPP. After 20 h of treatment cells were starved of Epo for another 20 h and then either lysed (for total cellular EpoR) or biotinylated for detection of surface proteins.

Fig. 4A demonstrates that either mevalonate or GGPP, but not FPP alone, prevents lovastatin's effects on EpoR surface expression. FPP + IPP, which are the precursors for GGPP could partially restore EpoR levels. To confirm that FPP and GGPP are taken up by cells and incorporated into protein, the ability of the two isoprenoids to prevent the effects of lovastatin on the farnesylation of Ras and geranylgeranylation of Rap1, respectively, was determined. Lovastatin treatment leads to the emergence of a slowly migrating upper band in the gel of the small GTPase which represents the unprenylated form of the protein. When FPP was added this band disappeared from the Ras blot and levels of Ras returned to control value. Similarly, GGPP addition made Rap1 band indistinguishable from that of the control (Fig. 4D). These results indicated that exogenous isoprenoids are taken up and utilized for protein prenylation in these cells.

In order to show specificity of lovastatin effect to EpoR, we used another cell system. Since ASE2 cells are not known to express any other receptor of the cytokine family besides EpoR, we used the 32 Da cell system and studied the expression of IL-3 receptor (IL-3R), a related member of the cytokine family. As shown in Fig. 4C, lovastatin treatment inhibited EpoR surface expression in 32 Da cells as it did in ASE2 cells. However, lovastatin treatment did not affect the surface levels of IL-3R. Furthermore, only mevalonate and GGPP were able to restore EpoR surface levels that were inhibited by lovastatin. These effects are similar to what was seen in ASE2 cells.

3.4. *Lovastatin-induced suppression of EpoR surface expression is partially due to inhibition of dolichol synthesis and glycosylation*

Since FPP + IPP/GGPP are utilized for both dolichol synthesis and post-translational modification of proteins through prenylation (Fig. 1), it is important to understand the importance of each of these processes for EpoR surface

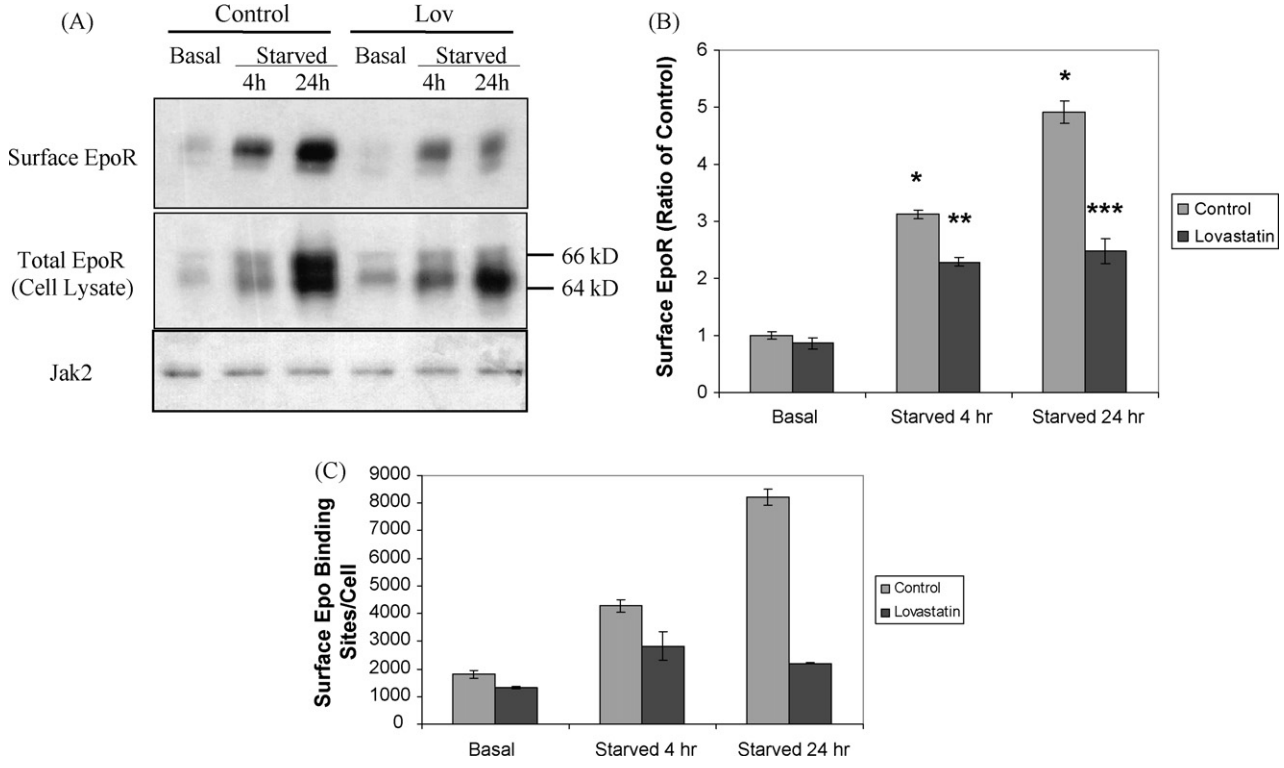

Fig. 3 – Mevalonate depletion suppresses EpoR expression on the cell surface. ASE2 cells were treated with 20 μM lovastatin for 20 h and then either starved for the indicated time points or left in Epo-nourished media (basal). Subsequently, cells were either lysed or subject to biotinylation of surface protein as described in Section 2. Western blots were then conducted for both groups using 7% SDS-PAGE and the indicated antibodies. Representative immunoblots are shown in (A) densitometric quantifications are graphed in (B) (means ± S.D. of triplicate samples from representative experiments expressed relative to basal control). Significance: *, $p < 0.05$ vs. basal control; **, $p < 0.05$ vs. 4 h starved control; ***, $p < 0.05$ vs. 24 h starved control as determined by unpaired two-tailed Student's *t*-test. (C) ASE2 cells were treated with 20 μM lovastatin for 20 h and then either starved for the indicated time points or left in Epo-nourished media (basal). Subsequently, cells were incubated with 3 nM [^{125}I]Epo for 3 h at 4 °C. Cells were then washed and radioactivity was measured in the cell pellet using gamma counter. Surface Epo binding sites were calculated as described in Section 2. Shown are means ± S.D. for duplicate experiments.

expression. To do so, EpoR glycosylation was first examined. Cells were cultured in the absence or presence of lovastatin for 24 h. Lovastatin cultures were additionally supplemented with 50 μg/ml dolichol. For the last 4 h of each treatment, radiolabeled glucosamine was added to cells. Cells were then lysed and EpoR immunoprecipitated. Radioactivity in the immunoprecipitates, which corresponds to degree of EpoR glycosylation, was measured.

Fig. 5A shows that lovastatin treatment leads to a decrease in EpoR glycosylation to 55% that of the control level. Dolichol reverses this effect but only to 80% of that of the control condition. Higher concentration of dolichol did not improve this result (data not shown). The effect of the glycosylation inhibitor, tunicamycin, on EpoR glycosylation was also studied. Tunicamycin inhibits EpoR glycosylation to levels comparable to those of lovastatin (Fig. 5A). However, tunicamycin seems to inhibit EpoR glycosylation in a manner that is different from that of lovastatin. Fig. 5B (left panel) shows that lovastatin inhibits the formation of the mature 66 kDa form of EpoR while tunicamycin induces downward shifts in both forms of EpoR. We also investigated the effect of lovastatin on total N-linked glycosylation in ASE2 cells. Fig. 5D shows that lovastatin inhibits total N-linked glycosylation. This process is at least partially dependent on dolichol, since adding dolichol to lovastatin-treated cells restores total glycosylation levels.

The effect of adding back dolichol on EpoR surface expression was also assessed (Fig. 5B and C). Again, dolichol only partially prevents lovastatin's effect on the cell surface expression of EpoR. Interestingly, tunicamycin treatment also decreased EpoR surface population, but to a lesser degree than did lovastatin.

3.5. *Inhibition of geranylgeranylation suppresses EpoR maturation and surface expression*

Since adding back dolichol only slightly reverses lovastatin's effect on EpoR surface expression we considered other mechanisms through which mevalonate depletion might explain lovastatin's effect. GGPP was able to completely restore EpoR surface levels and is a precursor that is utilized for post-translational modification of small GTPases through geranylgeranylation (Fig. 1). We investigated whether specifically inhibiting this process would mimic lovastatin's effect on EpoR surface presentation. Cells were treated with a

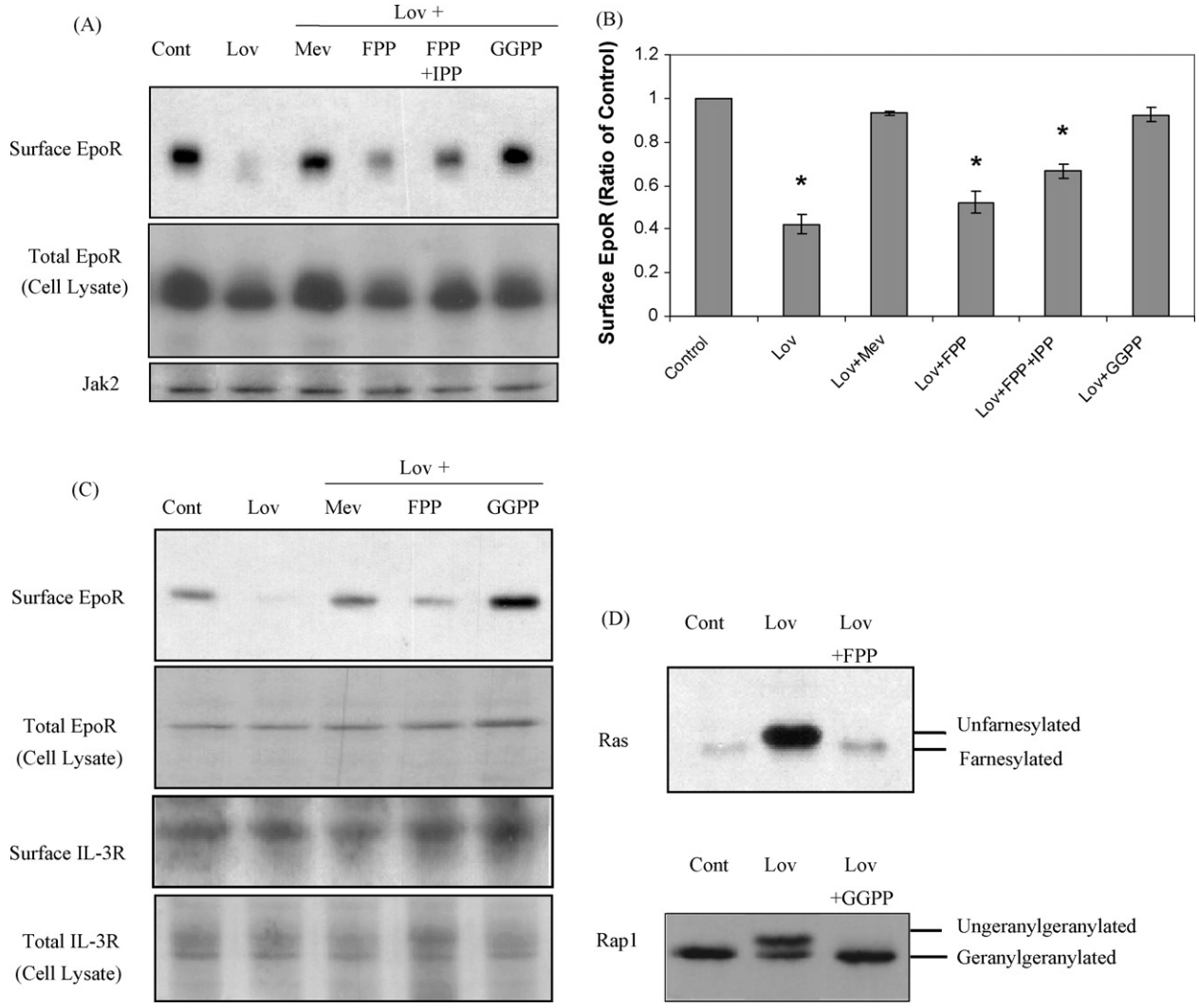

Fig. 4 – Mevalonate and GGPP prevent lovastatin's effects on EpoR. (A) ASE2 cells were treated with 20 μM lovastatin for 20 h. Lovastatin cultures were additionally supplemented with one of the following: 5 mM mevalonate, 10 μM FPP, 10 μM FPP plus 30 μM IPP, or 10 μM GGPP. Cells were then Epo-starved for 20 h in the presence of each treatment. Subsequently cells were either lysed or subject to biotinylation of surface proteins as described in experimental procedures. Western blots were then conducted for both groups using EpoR and Jak2 antibodies. (B) Densitometric quantifications of triplicate independent experiments (means ± S.D. expressed relative to control). Significance: *, $p < 0.05$ vs. control as determined by unpaired two-tailed Student's t-test. (C) 32 Da cells were treated with the indicated treatments for 24 h and then processed as in A. Western blots were conducted using EpoR or IL-3R antibodies. (D) As a positive control for FFP and GGPP functions the indicated cell lysates from A were run on 15% SDS-PAGE and immunoblotted with pan-Ras or Rap1 antibodies.

selective geranylgeranyl transferase inhibitor, GGTI-286, for 24 h. Cells were then starved and the surface population of EpoR was measured using the biotinylation experiment. Fig. 6 demonstrates that GGTI treatment leads to a decrease in EpoR surface expression in ASE2 cells in a concentration-dependent manner. This effect is not associated with any decrease in Jak2 protein levels (Fig. 6).

3.6. *Lovastatin suppresses Epo stimulatory effects on cell viability*

Finally, we wished to examine the effect of mevalonate depletion on the dose–response curves of Epo in ASE2 cells. We chose the MTT assay as a measure of Epo response since these cells are dependent on Epo for survival and proliferation. The Epo effect was assessed at 48, 72, and 96 h since these cells show signs of Epo starvation starting at 48 h [37]. As shown in Fig. 7A, mevalonate depletion downshifts the dose–response curve of Epo and attenuates the maximum response to Epo. Forty eight-hour treatment with lovastatin leads to a 35% decrease in the maximum response to Epo. This effect is consistent with our finding that lovastatin decreases EpoR surface expression (Fig. 3).

We also evaluated the effect of adding back mevalonate, FPP, or GGPP to lovastatin-treated cells and the effect of tunicamycin and GGTI on cell survival after 48 and 72 h treatment. As shown in Fig. 7B, adding back mevalonate or GGPP, but not FPP, reverses lovastatin's effect. Tunicamycin

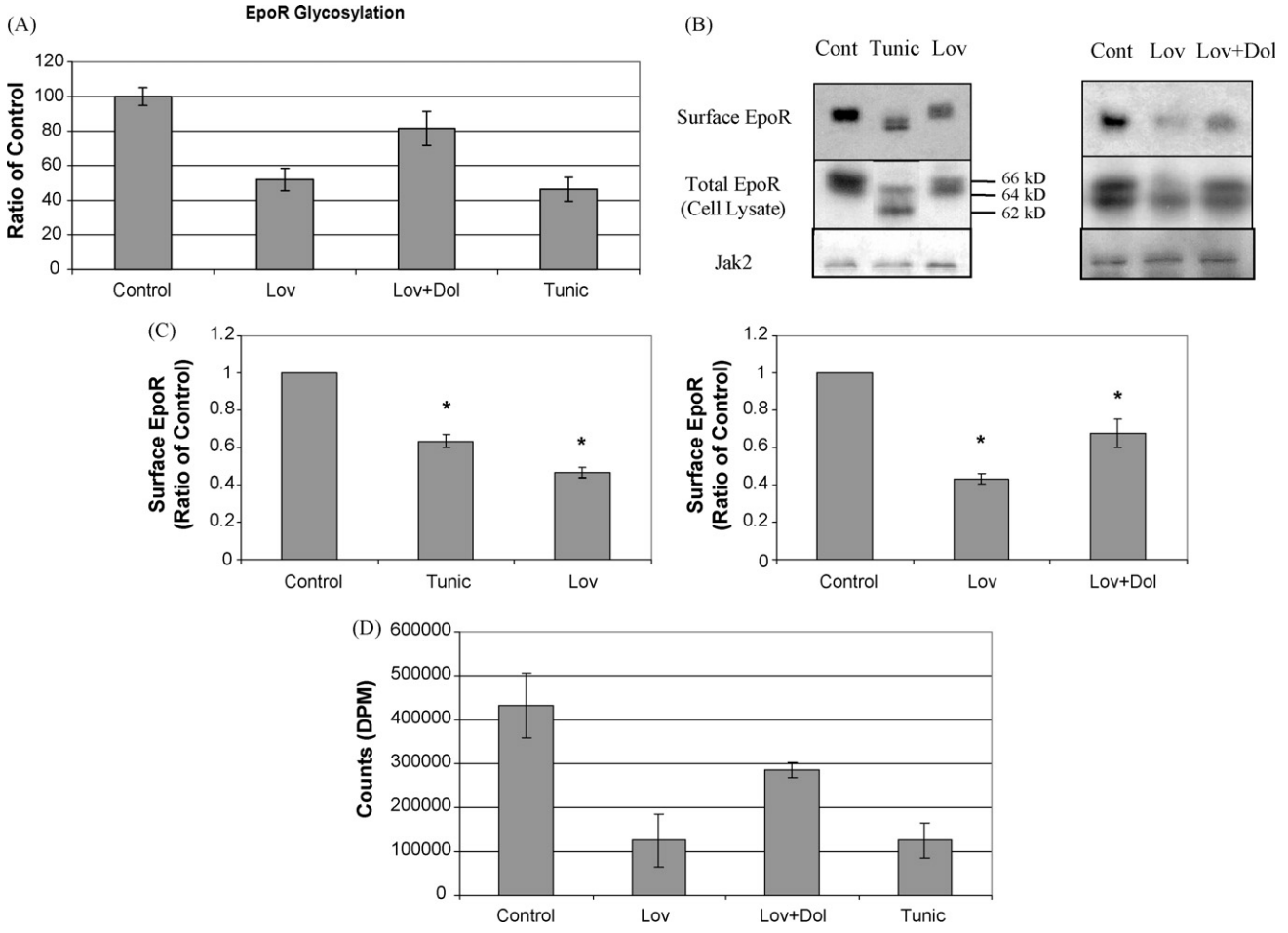

Fig. 5 – Lovastatin's effects are partially due to inhibition of dolichol synthesis and glycosylation. (A) ASE2 cells were treated with 20 μM lovastatin, lovastatin and 50 μg/ml dolichol, or 3 μg/ml tunicamycin for 24 h. During the last 4 h of each treatment cells were labeled with 20 μCi/ml [^{3}H]glucosamine. Glycosylation of EpoR was measured as described in experimental procedures. Results are expressed as means ± S.D. for triplicate experiments. The absolute control value is 1770 dpm/mg protein. (B) ASE2 cells were treated with 20 μM lovastatin, 3 μg/ml tunicamycin or lovastatin and 50 μg/ml dolichol for 20 h. Cells were then Epo-starved for 20 h. Subsequently cells were either lysed or subject to biotinylation of surface proteins as described in experimental procedures. Western blots were then conducted for both groups using EpoR and Jak2 antibodies. (C) Densitometric quantifications of triplicate independent experiments (means ± S.D. expressed relative to control). Significance: *, $p < 0.05$ vs. control as determined by unpaired two-tailed Student's t-test. (D) ASE2 cells were treated with 20 μM lovastatin, lovastatin and 50 μg/ml dolichol, or 3 μg/ml tunicamycin for 40 h. During the last 4 h of each treatment cells were labeled with 10 μCi/ml [^{3}H]glucosamine. Total N-linked glycosylation was measured as described in Section 2. Results are expressed as means ± S.D. for triplicate experiments.

treatment also inhibits survival of these cells though to a lesser degree than lovastatin. Inhibition of geranylgeranylation using GGTI-286 inhibited survival in a dose and time-dependent manner.

In order to show that the effects of lovastatin on cell survival are actually due to inhibition of Epo response we had to revert to 32 Da cells which express IL-3R. We examined the effect of lovastatin on cells grown in Epo-nourished media compared to cells grown in IL-3-nourished conditions. Interestingly, lovastatin decreased the survival of cells grown in Epo to a much further extent compared to cells grown in IL-3 (Fig. 7C). These results suggest that selective inhibition of Epo response is responsible for the effect of lovastatin on cell survival.

4. Discussion

Studies on EpoR have gained popularity recently because of the new findings that EpoR is expressed on the surface of many non-hematopoietic cancer cells and that this receptor is functional and contributes to the migration and invasion abilities of these cells [15,16]. Thus understanding the factors that govern EpoR surface presentation would provide clues on how to manipulate its expression and function.

We previously demonstrated in 32 Da cells that lovastatin inhibits signaling through EpoR by depletion of GGPP. Although these cells were stably transfected with EpoR, it is critical to ascertain the extent to which additional factors might alter surface presentation of EpoR in cells that

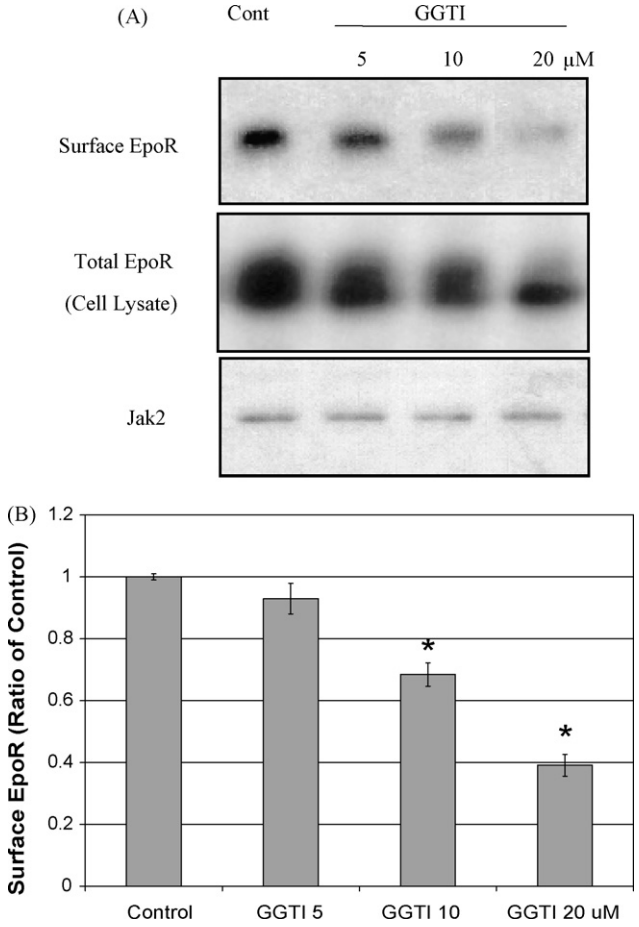

Fig. 6 – Inhibition of geranylgeranylation suppresses EpoR surface expression. Cells were treated with the indicated concentration of GGTI-286 for 20 h. Cells were then Epo-starved for 20 h. Subsequently cells were either lysed or subject to biotinylation of surface proteins as described in Section 2. Western blots were then conducted for both groups using EpoR and Jak2 antibodies. Representative immunoblots are shown in (A) densitometric quantifications are graphed in (B) (means ± S.D. of triplicate samples from representative experiments expressed relative to control). Significance: *, $p < 0.05$ vs. control as determined by unpaired two-tailed Student's t-test.

endogenously express EpoR. We therefore investigated the factors that control the expression and maturation of endogenous EpoR in a human erythroleukemia cell line that is strongly dependent on Epo for survival and growth-ASE2. We have shown that the widely used drug, lovastatin, inhibits the maturation of EpoR to the fully glycosylated, higher molecular weight form of EpoR and attenuates its expression on the cell surface, especially upon Epo starvation. This effect seems to be due to inhibition of two pathways by lovastatin; glycosylation and geranylgeranylation.

EpoR is first glycosylated in the ER to generate the 64 kDa form. This form matures to the 66 kDa with a complex Golgi-processed endoglycosidase H-resistant oligosaccharide. Epo starvation leads to an increase in both forms of EpoR in a time-dependent manner, which is attributed to inhibition of degradation and activation of protein synthesis [20,39] while mevalonate depletion inhibits the formation of the mature form without affecting the increase in the other form. However, it is notable that mevalonate depletion appears to decrease total EpoR levels and this is presumed to be due to the rapid turnover of the EpoR that is accumulating in the ER and not reaching the cell surface. The requirement for mevalonate is actually due to a specific requirement for two of its downstream products, dolichol and GGPP. We have shown that the two processes that are crucial for the transition from the 64 kDa form to the 66 kDa form of EpoR are glycosylation and geranylgeranylation. Dolichol is the carrier of N-linked oligosaccharides that is synthesized through the mevalonate pathway. Dolichol is also thought to play a role in the processing of the oligosaccharides in the Golgi apparatus [31,32]. The finding that dolichol is present in high concentrations in the Golgi apparatus supports this notion. Lovastatin treatment decreases glucosamine incorporation into EpoR immunoprecipitates. This effect is at least partially reversed by dolichol (Fig. 5). Furthermore, lovastatin inhibits total N-linked glycosylation and this effect is also dependent on dolichol (Fig. 5D). Lovastatin treatment appears to be distinct from the effect of the glycosylation inhibitor, tunicamycin. Unlike tunicamycin, lovastatin does not seem to inhibit the glycosylation of the 62 kDa form of EpoR to become the 64 kDa form. This might be a result of differences in the half-lives of dolichol in the different subcellular compartments with Golgi dolichol having a shorter half-life than ER dolichol. Alternatively, the second glycosylation step might be inhibited by a mechanism that is not completely dependent on dolichol. For example, lovastatin could be inhibiting the second glycosylation indirectly through inhibiting the translocation of EpoR from the ER to the Golgi where the second glycosylation takes place. This is consistent with the finding that dolichol treatment neither totally restore glycosylation nor surface expression of EpoR.

The effect of the geranylgeranylation inhibitor, GGTI-286, seems to agree with this hypothesis. Like lovastatin, inhibition of geranylgeranylation leads to suppression of the formation of the mature EpoR form and its expression on the cell surface. Many geranylgeranylated proteins play important roles in the process of membrane trafficking. For example, members of the Rho family were shown to be localized to the Golgi apparatus where they are involved in vesicle trafficking [40]. Our results suggest that inhibition of geranylgeranylation contributes majorly to lovastatin effects on EpoR. This is based on several findings: GGPP can fully restore EpoR surface levels that were inhibited by lovastatin, GGTI-286 inhibits surface EpoR expression in a manner that is similar to lovastatin, and GGTI-286 treatment mimics lovastatin effect on ASE2 cell survival. However, our results also suggest that dolichol levels and glycosylation become rate-limiting for EpoR surface expression under circumstances of GGPP depletion.

In a recent paper, it was shown that geranylgeranyl transferase inhibitors, including GGTI-286, inhibit proteasomal activity [41]. If this was the case, it would be expected for GGTI-286 to enhance surface EpoR expression because the proteasome is involved in EpoR degradation [20,39]. However, our results show the opposite effect for GGTI-286 (Fig. 6),

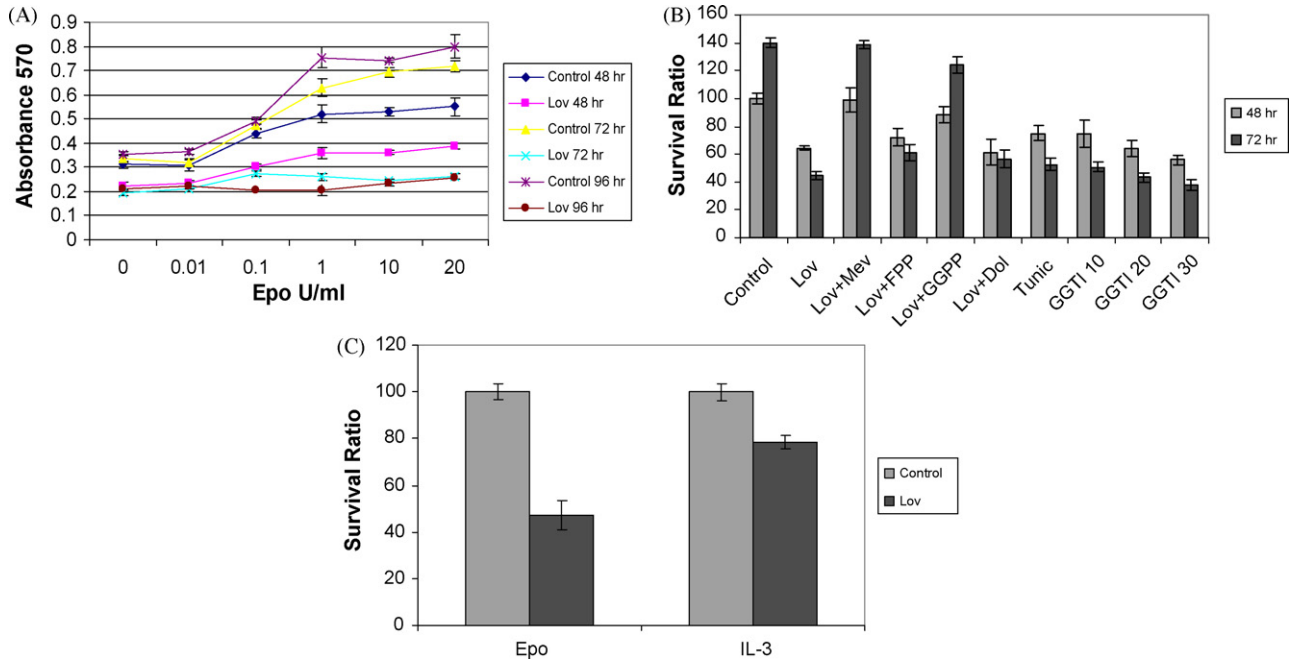

Fig. 7 – Mevalonate depletion attenuates the response to Epo. (A) ASE2 cell survival in response to Epo was used as a measure of Epo response. ASE2 cells were incubated in the presence of the different concentrations of Epo (U/ml, on the *x*-axis) for the indicated time points with or without 20 μM lovastatin. During the last 4 h of each treatment MTT solution (5 mg/ml) was added. MTT stop solution was then used for solubilization. Color intensity was measured at 570 nm using plate reader. Results are expressed as means ± S.E. for quadruplicate experiments. (B) ASE2 cells are incubated with the indicated treatments for 48 or 72 h in the presence of 10 U/ml Epo. Cell survival was measured using MTT assay as described in (A). Results are expressed as means ± S.E. for quadruplicate experiments. (C) 32 Da cells were treated with 20 μM lovastatin for 24 h in the presence of 10 U/ml Epo or IL-3. Cell survival was measured using MTT assay as described in A. Results are expressed as means ± S.D. for quadruplicate experiments.

which indicates that this agent is acting through a different mechanism.

Previous studies have shown that inhibition of glycosylation by tunicamycin treatment leads to a decrease in the total number of Epo binding sites [42]. However, other studies suggested that glycosylation is not required for EpoR surface expression and signal transduction [42,43]. These studies used transfected forms of EpoR and not endogenous EpoR. In many cases, transfected EpoRs behave in a manner that is different from endogenously expressed EpoR. In fact, when we used 32 Da cells we were unable to detect the different glycosylated forms of EpoR on Western blots (Fig. 4C) and tunicamycin treatment did not seem to affect EpoR migration on the gel in these cells (data not shown).

Jak2 is required for normal processing and cell surface expression of EpoR on the surface [38]. We did not detect any effect of lovastatin on Jak2 levels in these cells. However, the hypothesis that different pools of Jak2 exist that might be affected by mevalonate depletion was not addressed in our current studies. On the other hand, Jak2 activity is required for EpoR degradation and inhibiting Jak2 leads to an increase in surface EpoR [20]. We have previously shown that mevalonate depletion inhibits Jak2 phosphorylation and activation by Epo. Whether this inhibition of Jak2 is the reason for decreased EpoR degradation in response to Epo (Fig. 2) or it is a consequence of decreased EpoR surface expression warrants further investigation.

In conclusion, our results indicate that EpoR surface expression depends on at least two processes, glycosylation and geranylgeranylation. These are closely interacting and tightly regulated through the mevalonate biosynthetic pathway.

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