FEBS Letters 441 (1998) 34–38 FEBS 21266

Isolated endosomes from quiescent rat liver contain the signal transduction machinery

Differential distribution of activated Raf-1 and Mek in the endocytic compartment

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Received 23 October 1998

Abstract In this study we identify the molecules involved in the MAPK signal transduction pathway (Ras, Raf-1, Mek, Mek-P and MAPK) in highly purified endosomal fractions isolated from rat liver. Biochemical analysis shows that only the early-sorting endocytic compartment contains activated Raf-1 and Mek. Finally, the exogenous administration of EGF led to redistribution of Raf-1 from the caveolin-enriched plasma membrane into the endosomes.

Key words: Endosome; Endocytosis; Caveolin; Rat liver; Epidermal growth factor; Raf-1

1. Introduction

Signal-transduction pathways are crucial to the regulation of protein and membrane trafficking. Of particular interest are those initiated by receptor tyrosine kinases at the plasma membrane that affect the molecular mechanisms of endocytosis [1,2]. In recent studies it has been demonstrated that caveolae are the plasma membrane stores for molecules involved in the signal transduction. The first evidence of a relationship between signal-transduction mechanisms and caveolae was proposed by the morphological studies of Strosberg [3], which demonstrated the clustering of G-protein associated receptors (muscarinic acetylcholine and β-adrenergic receptors) in caveolae after agonist induction. More recently a number of signal transduction-related proteins have been found enriched in caveolae (G-proteins, PKC, IP_3 kinase, Ca^{2+} ATPase, non-receptor tyrosine kinase (NRTK), or G-protein associated receptors). Accordingly, various methods have also demonstrated the sensitivity of caveolae components to growth factors (EGF in fibroblasts and PDGF in endothelial and in NRK cells) [4–6] and to insulin in adipocytes [7].

In addition, direct interaction between lipid-modified signaling proteins and caveolin (G-protein α-subunits, Src-family tyrosine kinases, H-ras, eNOS and SHC) has been demonstrated (for a recent review see Anderson [8]). Interestingly, the direct binding of caveolin with lipid-modified signalling proteins seems to take place with the inactive form of those proteins; 20 residues were defined at the amino terminal region as caveolin 'scaffolding', as they bind the inactive form of the target protein [9].

In liver cells, it was shown that the endocytic compartment might be a possible alternative scenario for the regulation of signal transduction pathways. In fact, down-regulation of the EGF receptor after EGF activation [10], or insulin receptor kinase phosphorylation after insulin administration [11], as well as downstream events [12] are just some of the processes regulated in the hepatic endocytic compartment. Although signal transduction is believed to be a cascade of interactions initiated at the plasma membrane but exerting its final effects in the nucleus, it has now become clear that certain signal-transduction pathways affect membrane trafficking; for example, insulin binding stimulates trafficking within the endocytic compartment [1].

In this work, using highly purified endosomes and a caveolae-enriched plasma membrane fraction, the differential distribution of signalling molecules was demonstrated. Furthermore, the results of this study show that in quiescent hepatocytes there is basal activity of Raf-1 and Mek restricted to the early/sorting endocytic compartment. A possible pathway by which activated molecules could proceed from the cell surface into the endocytic compartment is discussed.

2. Materials and methods

2.1. Antibodies

Monoclonal mouse anti-EGF-R (E-3138) was from Sigma Chemical (Madrid, Spain). Rabbit anti-caveolin-1 (C13630), mouse anti-c-Raf-1 (R19120) and mouse anti-Mek-1 (M17020) were from Transduction Laboratories (Lexington, KY, USA). Anti-HGF-R (m-met) (SP260) (Cat. # sc-162) was from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal antibody anti-pan-Ras (Ab-2) (Cat. # OP22L) was from Oncogene Science (Uniondale, NY, USA). Rabbit anti-phospho-specific Mek1/2 antibody (Ser-217/221)(#9121S) and rabbit anti-phospho-specific MAPK antibody (Thr-202/Tyr-204)(#9101) were from New England Biolabs (Beverly, MA, USA). Monoclonal anti-MAPK (03-6600) was from Zymed Laboratories (San Francisco, CA, USA). Secondary antibodies HRP-conjugated were from Bio-Rad Laboratories (Barcelona, Spain).

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Abbreviations: CEF, caveolin-enriched plasma membrane fraction; CURL, compartment of uncoupling of receptors and ligands (early-sorting endosomes); EGF-R, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; Gαi, α-subunit of heterotrimeric protein G; GDI, Rab GTP dissociation inhibitor; GRB2, growth factor receptor binding protein 2; HGF-R (m-met), hepatic growth factor receptor; LDL, low density lipoprotein; Mek, MAPK/ERK kinase; mSOS, the mammalian homologue of SON OF SEVENLESS; MVB, multivesicular bodies (late endosomes); NRTK, non-receptor tyrosine kinase; PDGF, platelet derived growth factor; RRC, recycling receptor compartment; SHC, EGF-R-associated protein

0014-5793/98/$19.00
PII: S0014-5793(98)01517-8

2.2. Animals

Male Sprague-Dawley rats weighing 200–250 g were kept under a controlled lighting schedule with 12 h dark period. All animals received human care in compliance with institutional guidelines. Food and water were available ad libitum. In some experiments 20 μg of EGF receptor-grade (Sigma Chemical) were intravenously (portal vein) injected 15 min before liver extraction.

2.3. Isolation of endosomes, plasma membrane and a caveolin-enriched fraction from rat liver

After three days of 17α-ethinyl estradiol treatment, as described to induce the expression of the low density lipoprotein receptors [13], rats were anaesthetised with diethyl ether, and human LDL (5 mg of protein) was injected into the femoral vein and then, 20 min later, livers were removed and homogenised in 0.25 M sucrose with protease inhibitors. The method used for the isolation of three endosomal fractions from rat liver is described elsewhere [14–16]. Three distinct endosomal fractions were obtained after centrifugation of a crude endosome fraction in a sucrose gradient: MVB at 8.24/19.3%, CURL at 19.3/28.81%, RRC at 28.81/36.37% and CEF at 36.37/46% (w/v) interfaces. Each fraction was collected and ice-cold water was added to render the fractions isotonic. The isotonic fractions were pelleted, resuspended in 0.9% NaCl and stored at −80°C.

2.4. Gel electrophoresis and Western blots

SDS-polyacrylamide gel electrophoresis of proteins was carried out in 10% or 12% polyacrylamide, as described by Laemmli (1970) [17]. For Western blotting, polypeptides (5 μg of protein per channel) were transferred electrophoretically at 60 V for 90 min at 4°C to Immobilon-P Transfer Membranes (Millipore) and antigens were identified using different antibodies diluted in TBS (Tris-buffered saline) containing 0.5% powdered skimmed milk and finally the reaction product was detected using the ECL system (Amersham, UK). Image analysis of Western blots was performed with a Bio-Image system (Millipore). The protein content of the samples was measured by the method of Bradford [18] using bovine serum albumin as standard or the method of Lowry [19] for immunoprecipitated samples.

2.5. Raf-1 immunoprecipitation and kinase assay

Immunoprecipitacions were performed as described by Morrison [20]. Sixty μg of CEF, RRC and CURL were solubilised in 300 μl of RIPA buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA) containing 1 mM PMSF, 1 mM aprotinin, 20 μM leupeptin and 5 mM sodium vanadate. To immunoprecipitate Raf-1 from fractions, 2.5 μg of anti-Raf-1 monoclonal antibody or 2.5 μg of a non-related monoclonal antibody (control) were first prebound to 20 μl protein G-Sepharose beads (Sigma Biochemical) in 1 ml of RIPA buffer for 1 h at room temperature. After washing the anti-Raf-coated beads in RIPA buffer twice, the three fractions were added and incubated for 2 h at 4°C. The immunoprecipitated complexes were washed three times in 1 ml of cold NP-40 lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA) containing the proteinase inhibitor mixture (the same as above), resuspended and incubated for 20 min at 25°C in 40 μl of kinase buffer containing 30 mM HEPES-Na, pH 7.4, 7 mM $MnCl_2$ (made fresh), 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 15 μM ATP, 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, UK) and 40 mg/ml pGST-MEK (Upstate Biotechnology, Lake Placid, NY, USA) inactive fusion protein. The samples were then electrophoresed on SDS-polyacrylamide gels and the gels were stained with Coomassie blue, dried and exposed to X-ray films at −80°C.

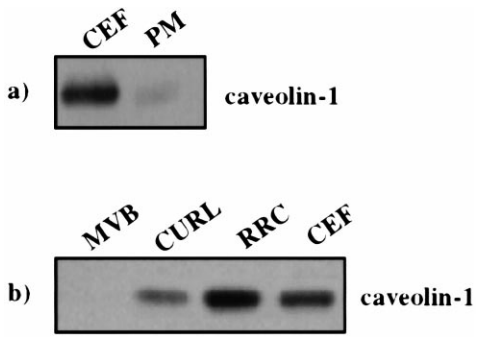

Fig. 1. Identification and distribution of caveolin-1 in plasma membrane and endosomal fractions from rat liver. Caveolin-1 (VIP-21) was identified in CEF and in CURL and RRC by Western blotting using an anti-caveolin antibody. a: Comparison between a plasma membrane fraction derived from the canalicular/lateral plasma membrane (PM) [24] and CEF, a plasma membrane fraction derived from the sinusoidal domain. b: Relative distribution of caveolin-1 between the three endosomal fractions and CEF. MVB, the late endosomes not containing detectable caveolin.

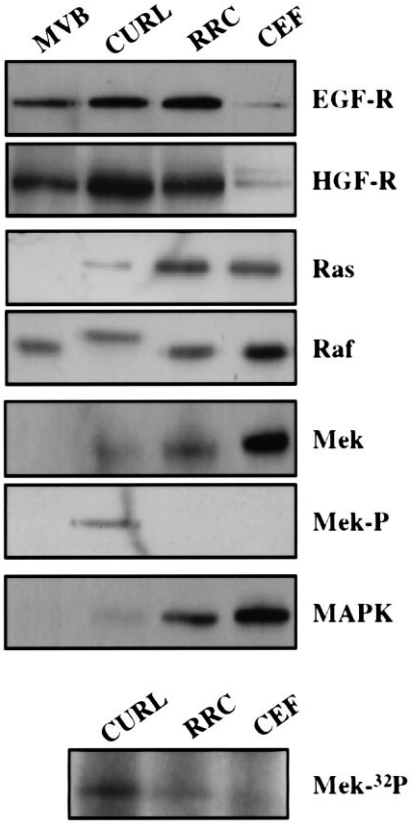

Fig. 2. Identification of signal transduction-related proteins in the three endosomal fractions and CEF. Signalling molecules of the MAPK pathway (EGF-R, HGF-R, Ras, Raf-1, Mek and MAPK) were identified in CEF and in endosomes by Western blot. The electrophoretical mobility of Raf-1 differs according to the fraction considered. Mek-P (substrate of activated Raf-1 kinase) was only present in early-sorting endosomes (CURL). Last panel shows the result of the kinase assay using pGST-Mek ([γ-^{32}P]ATP) and Raf-1 immunoprecipitated from CURL, RRC and CEF. Only Raf-1 immunoprecipitated from CURL was able to phosphorylate pGST-Mek.

3. Results and discussion

Highly purified rat liver endosomes have been morphologically and biochemically characterised [16,21,22]. Here we demonstrate that although signal transduction machinery is pre-organised in specialised microdomains at the plasma membrane it is recruited into the early/sorting endocytic compartment where Raf-1 and Mek are functional.

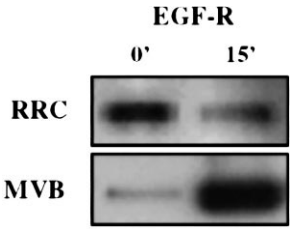

Fig. 3. Expression of EGF-R in the recycling receptor compartment (RRC) and late endosomes (MVB) after EGF administration. RRC and MVB endosomes from control rats (0′) or isolated after 15 min of EGF administration (15′) were electrophoresed and the expression of EGF-R studied by Western blotting. In endosomes from control liver EGF-R constitutively recycles between the sinusoidal plasma membrane and the early-sorting endosomes through the structures contained in RRC. However, after a saturating dose of EGF, the complex EGF/EGF-R is transported towards degradation and therefore detected in multivesicular bodies (MVB at 15′) with a concomitant decrease in RRC.

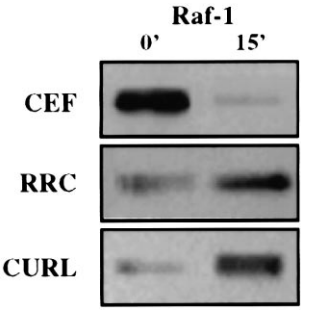

Fig. 4. Raf-1 expression in endosomal and caveolin-enriched plasma membrane (CEF) fractions after EGF administration. Endosomal and CEF fractions from control rats (0′) or isolated 15 min after EGF administration (15′) were electrophoresed and the expression of Raf-1 studied by Western blotting. After EGF injection there was a decrease in the levels of Raf-1 detected in the sinusoidal plasma membrane fraction (CEF), whereas the amount of Raf-1 in the endosomal fractions (RRC and CURL) increased.

3.1. Compartmentalised signal transduction machinery in the hepatocyte

The view that caveolae are plasmalemmal microdomains specialised in signal transduction processes is under very active research; however, information regarding the hepatic cell is scarce and controversial [23]. Highly purified endosomes and two different plasma membrane fractions from rat liver were used in this study. Fig. 1 shows the subcellular distribution of caveolin, by Western blotting, in plasma membrane (PM[1] and CEF[2]) and in endosomal fractions (MVB, CURL and RRC); caveolin was enriched in RRC and CEF whereas it was not detected in the late endosomes. In addition, using the same endosomal fractions and CEF the distribution of EGF-R, HGF-R (m-met), Ras, Raf-1, Mek, Mek-P and MAPK was studied (Fig. 2). Previous studies have shown the presence, in the same endosomal fractions, of other related signalling molecules: PKCα, Gαi and Ca^{2+} ATPase [22].

Although Raf-1 is enriched in CEF, it is present in all studied fractions; interestingly, the electrophoretic mobility of Raf-1 differs according to the fraction considered. In CEF and RRC, Raf-1 shows a higher mobility than CURL; while in MVB it shows an intermediate mobility. Changes in the mobility have been associated with different phosphorylation states, which in turn are the expression of its activity. In order to determine whether any of the Raf-1 detected in the fractions was active, two experimental approaches were followed. First, the search for its active substrate, Mek-P, was conducted; this kinase was exclusively present in early endosomes (while Mek was also present in CEF and in RRC). Secondly, a kinase assay was conducted using GST-Mek as an exogenous substrate and Raf-1 immunoprecipitated from each of the endosomal or CEF fractions (Fig. 1; Mek-^{32}P). Thus, the results obtained were in agreement with Western blotting and showed that the only Raf-1 able to phosphorylate Mek was the one immunoprecipitated from CURL. Therefore, caveolae might be stores were signal transduction machinery is pre-organised whereas the endocytic compartment is the target organelle for the recruitment and activation. In fact, the relationship between the endocytic compartment and signal transduction pathways was also demonstrated by Di Guglielmo et al. [12] using a Golgi-endosomal fraction from rat liver.

3.2. EGF induces the internalisation of pre-organised transduction machinery to endosomes

It has been reported that after administration of a single receptor-saturating dose of EGF there is a rapid and extensive receptor internalisation with a concomitant tyrosine phosphorylation of 170- and 55-kDa polypeptides and the association of SHC and GRB2 adapter proteins with the EGF-R; then, the SH3 domains of GRB2 interact with mSOS [12]. The direct interaction of mSOS with $p21^{ras}$ promotes the activation of MAP kinase via Raf-1 and Mek. To study the effect of EGF administration in this system, CEF and endosomes were isolated from treated rats and analysed. Firstly, internalisation and intracellular processing of EGF were evaluated by means of its receptor EGF-R (Fig. 3). In quiescent liver EGF-R recycles between the plasma membrane and the endocytic compartment [10]. After EGF binding the complex EGF/EGF-R was internalised and was transported to lysosomes through the late endosomal compartment (Fig. 3). The effect of EGF administration on the expression of Raf-1 was also studied. Fig. 4 shows a progressive redistribution of Raf-1 from the

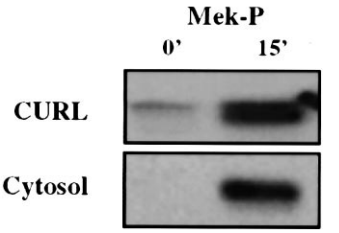

Fig. 5. Mek-P expression in early-sorting endosomes (CURL) and in cytosol after EGF administration. Early-sorting endosomes (CURL) and cytosol were isolated from control animals (0′) or obtained 15 min after EGF administration (15′); samples were electrophoresed and the expression of Mek-P studied by Western blotting. The amount of Mek-P increased in early-sorting endosomes (CURL) and in the cytosol after EGF injection.

[1] PM is a plasma membrane fraction derived from the canalicular and lateral plasma membrane domains of the hepatocyte [24].

[2] CEF is a caveolin-enriched plasma membrane fraction derived from the sinusoidal plasma membrane domain (Pol and Enrich, unpublished).

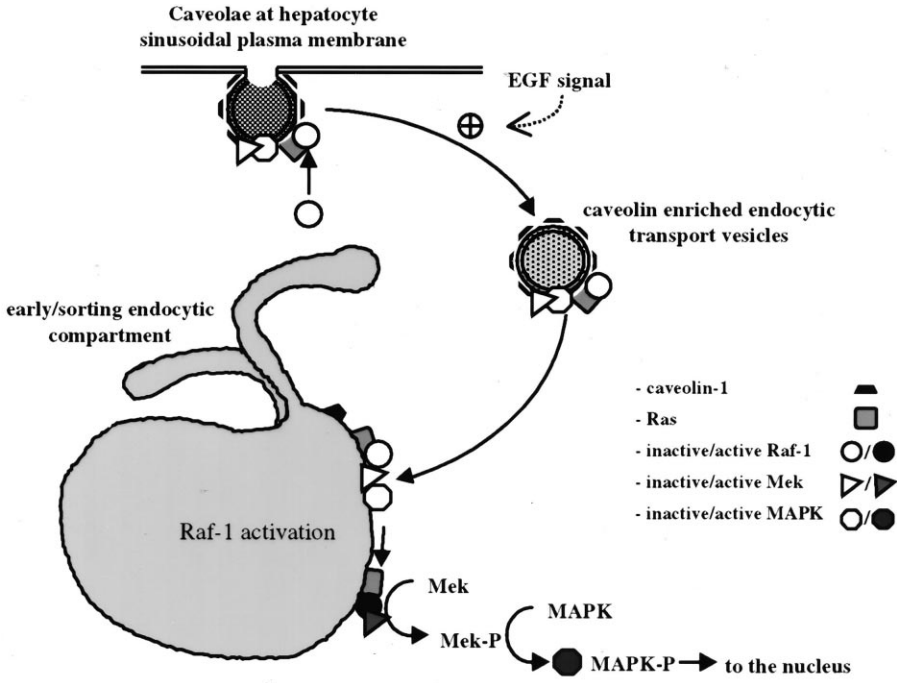

Fig. 6. Caveolin pathways in the hepatocyte. The model depicts the transport of caveolin and the signal transduction machinery from the caveolae at sinusoidal plasma membrane to the early/sorting endocytic compartment through intermediate structures (see text for details).

caveolin-enriched plasma membrane fraction (CEF) into the different endocytic compartments (RRC and CURL) after 15 min.

Finally, the expression of Mek-P was also studied. Fig. 5 shows that the levels of activated Mek-P increased in the early-sorting compartment (CURL at 15′) in response to EGF. After EGF administration, Mek-P was also detected in cytosol, but not in other endocytic or membrane fractions (not shown). These results indicate that EGF triggers the internalisation of pre-organised transduction machinery from plasma membrane domains enriched in caveolin into endosomes with a concomitant increase in activated forms of Raf-1 and Mek.

3.3. From caveolae to early endosomes

A model is postulated that predicts the recruitment of the Ras-GTP/Raf-1/Mek/MAPK complex from the caveolae into the early/sorting endocytic compartment where the phosphorylation of Raf-1, Mek and eventually of MAPK should be achieved (Fig. 6).

The homology of caveolin ('scaffolding' region) with GDI protein might explain that interaction of caveolin/Ras – and the concentration of Ras in caveolae independently of EGF mediated signal – could keep Ras inactive. Binding of growth factors, such as EGF, could induce the dissociation of the complex caveolin/Ras and allow accessibility of Ras to GTP; Raf-1 may then interact with Ras-GTP at the membrane of caveolae and induce the recruitment of Ras-GTP/Raf-1 into the endocytic compartment. It has been demonstrated that although the interaction of the complex Ras-Raf-1 is necessary for the recruitment of Raf-1 to the membrane, this event is not sufficient to activate the kinase activity of Raf-1. The activation of Raf-1 kinase is poorly understood. Approaches in vitro demonstrated that KSR kinases (kinase suppressor of Ras, related to post-inflammatory processes), PKC (α, δ, ε), members of Src and JAK-2 (Janus kinase 2) may phosphorylate and activate Raf-1 [25].

In conclusion, for the first time in the hepatic cell, it is shown that the signal transduction machinery is restricted (pre-organised) in specific domains at the cell surface enriched in caveolin. The presence of active Raf-1 and Mek-P in endosomes isolated from quiescent livers demonstrates that there is a basal activity of signal transduction pathways. While caveolin at the cell surface may function as a scaffolding protein to sequester or localise caveolin-interacting proteins, such as Ras or Gαi, intracellular caveolin might be involved in shuttling the signal transduction molecular machinery into the sorting endocytic compartment.

Acknowledgements: This work was supported by PM96-0083.

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