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Heterotrimeric Gαq11 Co-Immunoprecipitates With Surface-Anchored GRP78 From Plasma Membranes of α_2M*-Stimulated Macrophages

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Abstract We have previously shown that a fraction of newly expressed GRP78 is translocated to the cell surface in association with the co-chaperone MTJ-1. Proteinase and methylamine-activated α_2M (α_2M*) bind to cell surface-associated GRP78 activating phosphoinositide-specific phospholipase C coupled to a pertussis toxin-insensitive heterotrimeric G protein, generating IP_3/calcium signaling. We have now studied the association of pertussis toxin-insensitive Gαq11, with GRP78/MTJ-1 complexes in the plasma membranes of α_2M*-stimulated macrophages. When GRP78 was immunoprecipitated from plasma membranes of macrophages stimulated with α_2M*, Gαq11, and MTJ-1 were co-precipitated. Likewise Gαq11 and GRP78 co-immunoprecipitated with MTJ-1 while GRP78 and MTJ-1 co-immunoprecipitated with Gαq11. Silencing GRP78 expression with GRP78 dsRNA or MTJ-1 with MTJ-1 dsRNA greatly reduced the levels of Gαq11 co-precipitated with GRP78 or MTJ-1. In conclusion, we show here that plasma membrane-associated GRP78 is coupled to pertussis toxin-insensitive Gαq11 and forms a ternary signaling complex with MTJ-1. J. Cell. Biochem. 104: 96–104, 2008.

Key words: monocytes/macrophages; cell surface molecules; signal transduction

α_2-Macroglobulin (α_2M) is a circulating pan-proteinase inhibitor which reacts with either proteinases, or small nucleophiles at an internal thiol ester, resulting in receptor-recognized forms of the inhibitor (α_2M*) [Krieger and Herz, 1994]. α_2M* binds to two cell surface receptors; namely, the low density lipoprotein receptor-related protein (LRP) and the more recently identified α_2M* signaling receptor (α_2MSR) [Misra et al., 1994a,b, 1995, 1999, 2002, 2004; Howard et al., 1996a,b; Asplin et al., 2000]. These two binding sites are significantly different with respect to the number of binding sites, binding affinities, and their modulation by RAP, Ni^{2+}, and bacitracin [Misra et al., 1994a,b, 1995, 1999, 2002, 2004; Howard et al., 1996a,b; Asplin et al., 2000]. We isolated α_2MSR from macrophages and 1-LN prostate cancer cells and identified it as cell surface-associated GRP78 [Misra et al., 2002]. The binding of α_2M* to GRP78, and not to LRP, elevates intracellular IP_3, $[Ca^{2+}]_i$, and cAMP levels [Misra et al., 1993, 1994a,b, 1995, 2002]. Incubation of cells with anti-GRP78 antibodies or silencing *GRP78* gene expression by RNAi greatly attenuates the binding of α_2M* to macrophages or 1-LN cells and prevents the increase in IP_3 and $[Ca^{2+}]_i$ observed upon α_2M* binding to these cells [Misra et al., 2002, 2004]. Treatment of cells with GTPγS before α_2M* stimulation further increases the levels of IP_3 and $[Ca^{2+}]_i$; in contrast, preincubation with GDPβS completely blocks these changes [Misra et al., 1994a,b, 1995, 1996, 1999; Misra and Pizzo, 2002]. Preincubation of cells with either cholera toxin or pertussis

Abbreviations used: GRP78, glucose-regulated protein of M_r ~78,000; MTJ-1, murine tumor cell DnaJ-like protein 1; α_2M, α_2-macroglobulin; α_2M*, the receptor recognized form of the inhibitor made by reaction with a proteinase or by direct attack by small amines on an internal thiol ester bond; LRP, the low density lipoprotein receptor-related protein; α_2MSR, the α_2M* original receptor; RAP, receptor-related protein which blocks the binding of all known ligands to LRP; IP_3, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; ECF, enhanced chemifluorescence.

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toxin demonstrates that α_2M*-induced increase in cAMP levels is coupled to a Gαs protein whereas α_2M*-induced increase in IP_3 and $[Ca^{2+}]_i$ is coupled to a pertussis toxin-insensitive G protein [Misra et al., 1993, 1994a,b, 1995]. Receptors coupled to Gαq mediate a wide range of cellular responses which include cell proliferation, neuronal signaling, and leukocyte activation. Ligand binding to growth factor receptors induces receptor tyrosine phosphorylation which transmits the extracellular message via recruiting the adaptor proteins Grb2/shc and the guanine nucleotide exchange factor sos. This results in the activation of the Ras/MAPK signaling pathway. Ras can also activate the PI 3-kinase signaling pathway via direct interaction with the catalytic subunit of PI 3-kinase [Lowy and Willumsen, 1983; Kolch, 2000]. Binding of α_2M* to GRP78 stimulates its tyrosine phosphorylation with subsequent activation of the Ras/MAPK and PI 3-kinase signaling pathways [Misra and Pizzo, 1998a,b, 2004; Misra et al., 2005c]. On the basis of mitogenic signaling and cellular growth promoting effects of α_2M*, we have suggested that α_2M* functions like a growth factor for macrophages, 1-LN prostate cancer cells, and rheumatoid synovial fibroblasts [Misra et al., 1993, 1994a,b, 1995, 1996, 1997, 1999, 2002, 2004, 2005c; Howard et al., 1996a,b; Misra and Pizzo, 1998a,b, 1999, 2002, 2004; Asplin et al., 2000].

G protein-coupled receptor signaling is the most conserved and ubiquitous signaling system in eukaryotes for transferring information across the plasma membrane to the intracellular environment [Gilman, 1987; Hamm, 1998; Marinissen and Gutkind, 2001; Neves et al., 2002; Breitwieser, 2004; Patel, 2004]. The binding of a ligand to cell surface receptors initiates conformational changes in receptor subdomains that transfer this signal to heterotrimeric G proteins on the inner face of the membrane. The heterotrimeric G proteins are comprised of α, β, and γ subunits (Gα, Gβ, and Gγ). In the resting state, the α subunit is bound to GDP in association with the Gβγ units which is coupled to the receptor [Gilman, 1987; Hamm, 1998; Marinissen and Gutkind, 2001; Breitwieser, 2004; Patel, 2004; Neves et al., 2002]. Upon ligand binding, receptor-mediated activation of heterotrimeric G proteins increases the rate of GDP-GTP exchange on the Gα subunit. The GTP-bound active Gα subunit dissociates from βγ subunits and both these moities activate their effectors, namely, adenylyl cyclase, PI-specific PLCβ and γ, and ion channels [Gilman, 1987; Hamm, 1998; Marinissen and Gutkind, 2001; Neves et al., 2002; Breitwieser, 2004; Patel, 2004]. The intrinsic GTPase activity of the Gα subunit hydrolyses GTP to GDP and thus terminates the signaling [Gilman, 1987; Hamm, 1998; Marinissen and Gutkind, 2001; Neves et al., 2002; Breitwieser, 2004; Patel, 2004]. The GTPase activity of the Gα subunit is also modulated by regulators of G protein signaling (RGS) as well as other effectors [Hamm, 2001; Milligan, 2004; Sato et al., 2004; Waters et al., 2004]. Based upon their sequence homology and sensitivity to toxins, the α subunit family is subdivided into Gαs, Gαi/o, Gαq11, and Gα12/13 isoforms [Gilman, 1987; Neves et al., 2002]. Some of the pleiotropic actions of the growth factors via their single membrane spanning protein kinase receptors also involve the activation of heterotrimeric G proteins [Guderman et al., 2000; Patel, 2004; Vazquez-Prdo et al., 2003]. For example, EGF activates ERK/MAPK signaling as well as phospholipase Cγ activity which is pertussis toxin-sensitive [Misra et al., 1995; Patel, 2004] in contrast to α_2M*-induced activation which is pertussis toxin insensitive [Misra et al., 1995]. EGF also elevates cAMP levels by activating adenylyl cyclase [Poppleton et al., 1996]. Insulin-dependent lipolysis and inhibition of glucose oxidation is blocked by pertussis toxin [Goren et al., 1985]. In adipocytes, insulin-elicited increase in GLUT4 translocation to the plasma membrane is Gαq11-dependent [Imamura et al., 1999]. PDGF and FGF growth factor receptors as well as integrin receptors signal via heterotrimeric G proteins [Patel, 2004].

GRP78 is essentially an ER resident protein; however, in certain situations a small pool of GRP78 in association with the co-chaperone MTJ-1, translocates to the plasma membrane where the complex remains docked in the intramembrane domain [Misra et al., 2005a]. We have shown that cell surface-associated GRP78 in α_2M*-stimulated cells exists in complex with PAK-2, and NCK, as well as with MTJ-1 [Misra et al., 2005b,c]. This multiprotein complex is involved in cellular responses, since inhibition of tyrosine phosphorylation, incubation of cells with anti-GRP78 antibodies, silencing expression of *GRP78*, *MTJ-1*, or *PAK-2* genes profoundly impairs the formation of this multiprotein complex and cellular responses [Misra et al., 2005b,c].

If GRP78 and MTJ-1 exist in the plasma membrane as envisaged above, then Gαq11 should be associated with the GRP78/MTJ-1 complex in α_2M*-stimulated cells. We have tested this hypothesis by silencing expression of the *GRP78* and *MTJ-1* genes employing RNA interference and examining macrophage plasma membranes for the presence of GRP78, MTJ-1, and Gαq11 from cells stimulated with α_2M*. We show here that GRP78, MTJ-1, and Gαq11 form a ternary signaling complex in the plasma membrane, since all three are co-precipitated with each other in the respective GRP78 and MTJ-1 immunoprecipitates.

MATERIALS AND METHODS

Animals and Reagents

The use of mice for these studies was approved by the Institutional Animal Use Committee in accordance with relevant federal regulations. Culture media were purchased from Invitrogen Life Technologies. Polyclonal antibodies against GRP78 were purchased from Stressgen. Polyclonal antibodies against Gq/α11 were purchased from Upstate Cell Signaling Solutions. Antibodies against caveolin were purchased from Cell Signaling Technologies. Antibodies against flotillin-1, and the heavy chain of clathrin, were procured from Santa Cruz. Antibodies against MTJ-1 were raised against the sequence beginning at residue 105, NH_2-LVAIYEVLKVDERRQRYVDVL-COOH, of MTJ-1 (SWISS-PROT, primary accession no Q61712) in rabbits (Genemed Synthesis) [Misra et al., 2005a,b]. α_2M* was prepared as described previously [Misra et al., 1994a,b]. Other reagents of the highest grade available were purchased locally.

Chemical Synthesis of dsRNAs Homologous in Sequence to the Target GRP78 and MTJ-1 Genes Respectively

The chemical synthesis of dsRNA homologous in sequence to the target GRP78 K_{521}NKITIT$_{527}$ peptide, (mRNA sequence 5′-AAG AAT AAA ATA ACA ATA ACA-3′; SWISS-PROT Primary accession number P11021) and target MTJ-1 K_{321} RQAPEW$_{327}$ peptide (mRNA sequence 5′-AAA AGA CAA GCA CCA GAA TGG-3′; SWISS-PROT Primary accession number Q61712) were performed by Ambion. For making the GRP78 dsRNA, the sense (5′-GAA UAA AAU AAC AAU AAC ATT-3′) and antisense (5′-UGU UAU UGU UAU UUU AUU CTT-3′) oligonucleotides were annealed according to the manufacturer's instructions. For making the MTJ-1 dsRNA, the sense (5′-AAG ACA AGC ACC AGA AUG GTT-3′) and antisense (5′-CCA UUC UGG UGC UUG UC U UTT-3′) oligonucleotides were annealed according to the manufacturer's instructions. Throughout the entire period of experimentation, handling of reagents was performed in an RNAse-free environment. Briefly, equal amounts of sense and antisense oligonucleotides were mixed in an annealing buffer and heated at 90°C for 1 min, then maintained for 1 h at 37°C in an incubator. The dsRNAs preparation was stored at −20°C.

Immunoprecipitation of Plasma Membrane Lysates With Anti-Gaq11, GRP78, or MTJ-1 Antibodies From Cells Treated With α_2M*, EGF, or Glucagons

Macrophages which had adhered for 2 h were incubated in the RPMI medium overnight as above. The monolayers were washed twice with HHBSS, a volume of above RPMI medium added and cells stimulated with buffer, α_2M* (50 pM/15 min), EGF (10 ng/15 min), or glucagon (20 μg/15 min), respectively. The cells were incubated at 37°C in a humidified CO_2 (5%) incubator. The reaction was terminated by aspirating the medium and the respective cells were processed for the isolation of plasma membranes as described above. These plasma membranes were lysed in the above lysis buffer and their protein contents determined. Equal amounts of lysate protein were immunoprecipitated with anti-GRP78, MTJ-1, or Gαq11 antibody, respectively. The immunoprecipitates were washed thrice with lysis buffer, a volume of sample buffer added, the samples boiled for 5 min, and then centrifuged. The samples were electrophoresed on gels (10%), transferred to membranes, and immunoblotted with antibodies against GRP78, MTJ-1, or Gαq11, respectively. The immunoblots were visualized by ECF in a Storm 860 phosphorimater (Amersham Biosciences).

Immunoprecipitation of GRP78, MTJ-1, and Gq/α11 in Plasma Membranes From Normal Cells and Cells Where the Expression of GRP78 or MTJ-1 Were Silenced

Thioglycollate-elicited peritoneal macrophages were obtained from pathogen-free 6-week old C57BL/6 mice (National Cancer

Institute) in buffer containing 10 mM HEPES (pH 7.4) and 3.5 mM $NaHCO_3$ (HHBSS). The cells were washed with HHBSS and suspended in RPMI 1640 medium containing 2 mM glutamine, penicillin (12.5 units/ml); streptomycin (6 μg/ml) and 5% FBS plated in 4- to 6-well plates and incubated for 2 h at 37°C in an humidified CO_2 (5%) incubator. The monolayers were washed three times to remove non-adherent cells and the monolayers were incubated overnight at 37°C in the above RPMI 1640 medium before study. The plasma membranes from cells in this study were prepared identically to those described recently [Misra and Pizzo, 1999, 2002]. Macrophages incubated overnight in 6-well plates (10×10^6 cells/well) were washed twice with HHBSS, a volume of the medium added and the cells stimulated in duplicates with α_2M* (50 pM/20 min) or buffer and incubated as above. The reaction was terminated by aspirating the medium, a volume of chilled HHBSS buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM PMSF, 10 μM benzamidine, and 10 μM leupeptin added and the cells scraped into chilled glass homogenizing tubes. Plasma membranes were isolated as described previously [Misra and Pizzo, 1999, 2002]. In some studies the expression of the *GRP78* or *MTJ-1* genes, respectively, were silenced prior to stimulation with α_2M*. Transfection was achieved with GRP78 dsRNA (25 μg/48 h) or MTJ-1 dsRNA (25 μg/48 h) as described previously [Misra et al., 2002, 2004, 2005a,b,c]. At the end of transfection period, the cell in 6-well plates (1×10^6 cells/well) were washed with medium and cells stimulated in quadruplicate with α_2M* (50 pM/20 min) or buffer and incubated as above. The reactions were terminated by aspirating the medium, a volume of chilled HHBSS buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM PMSF, 10 μM benzamidine, and 10 μM leupeptin added and cells scraped into chilled glass homogenizing tubes and plasma membrane isolated as described [Misra and Pizzo, 1999]. The plasma membranes were lysed in lysis buffer containing 20 mM Tris–HCl (pH 8.6), 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 1 mM PMSF, 20 μg/ml leupeptin, and 0.5% NP 40 on ice for 10 min and the protein contents of lysates determined [Bradford, 1976]. Equal amounts of plasma membrane protein lysates from macrophages treated with either buffer or α_2M* (50 pM/20 min) were immunoprecipitated with anti-GRP78 (1:50), MTJ-1 (1:20), or Gαq11 antibodies, respectively, as described [Misra et al., 2005a,b]. To control for the specificity of the antibodies employed to precipitate GRP78, MTJ-1, and Gαq11, plasma membrane lysates from buffer or α_2M*-stimulated cells were immunoprecipitated with a non-immune rabbit IgG (whole molecules; 4 μg/ml). The respective immunoprecipitates were washed three times with lysis buffer, a volume of sample buffer was added and the samples were boiled for 5 min and centrifuged. The preparations were then electrophoresed on gels (10%), transferred to membranes, immunoblotted with antibodies against GRP78 (1:20,000), MTJ-1 (1:500), or Gq/α11 (1:1,000), respectively, and visualized by ECF in a Storm 860 Phosphorimager® (Amersham Biosciences).

Co-Localization of Caveolin and Flotillin, But Not Clathrin, With GRP78 in Plasma Membranes of α_2M*-Stimulated Macrophages

Experimental details of isolating plasma membranes, immunoprecipitation of GRP78, and processing of immunoprecipitates for electrophoresis, and membrane transfer were as described above except that the respective membranes were immunoblotted with anti-caveolin (1:100), flotillin (1:100), or clathrin (1:800, 1:1,000, or 1:1,500) antibodies, respectively and protein bands visualized by ECF using a phosphorimaging.

RESULTS

GRP78 in Plasma Membrane Exists in Association With Heterotrimeric Gαq11 and the Co-Chaperone MTJ-1

Binding of α_2M* to GRP78 on the cell surface triggers MAPK and PI 3-kinase signaling which is coupled to activation of a pertussis toxin-insensitive G protein. In the first series of studies we demonstrated that immunoprecipitation of GRP78 was accompanied by the co-precipitation of MTJ-1 and Gαq11 (Fig. 1). We have previously demonstrated that MTJ-1 co-precipitates with GRP78 [Misra et al., 2005a], but this is the first direct demonstration that Gαq11 is associated with the GRP78/MTJ-1 complex. Likewise GRP78 and Gαq11 co-immunoprecipitated with MTJ-1 while GRP78 and MTJ-1 co-immunoprecipitated with GRP78 and Gαq11 (Figs. 2 and 3). As a control, non-immune

IgG was employed. While these immunoprecipitates contain IgG, no GRP78, MTJ-1, or Gαq11 were found under the experimental conditions (Fig. 1B). To examine the specificity of Gαq11 antibodies under our experimental conditions, we immunoprecipitated plasma membrane lysates from glucagon or EGF treated cells. The results show that Gαq11 is immunoprecipitated only by antibodies directed against GRP78 or MTJ-1 and vice versa in α_2M*-treated cells (Figs. 2 and 3). These results thus confirm that MTJ-1 is associated with GRP78 at the plasma membrane. Furthermore, GRP78, MTJ-1, and Gqα11 exist as a signaling complex in the plasma membrane of α_2M*-stimulated cells.

Silencing of the Expression of GRP78 or MTJ-1 by RNAi Disrupts Gαq11 Association With GRP78 and MTJ-1

Incubation of cells with anti-GRP78 antibodies before α_2M* stimulation greatly inhibits cell surface binding of α_2M*, the concomitant activation of PLCβ, the resultant increase in intracellular IP_3 and $[Ca^{2+}]_i$, and PI 3-kinase activation [Misra et al., 2002, 2004]. This results in downregulation of the ability of α_2M* to promote DNA synthesis and cellular proliferation. Similar results were observed when the expression of either the GRP78 or MTJ-1 gene were silenced by RNAi [Misra et al., 2005a,b]. In our earlier experiments the evaluation of the degree of *GRP78* and *MTJ-1* gene silencing with their respective dsRNAs under our experimental conditions was performed by quantifying both protein and mRNA levels, respectively. In both cases we consistently observed a reduction in the protein and mRNA levels of 60–70%, respectively [Misra et al., 2005a,b, 2006]. In the next series of experiments we employed RNAi to inhibit expression of the GRP78 and MTJ-1 genes, respectively. By limiting the availability of these two plasma membrane proteins, Gαq11 should also be decreased in the GRP78 immunoprecipitate of plasma membranes from α_2M*-stimulated cells as compared to the membranes from control cells. Consistent with this hypothesis, silencing of either GRP78 or MTJ-1 expression not only greatly diminishes the presence of these proteins in the plasma membrane protein fraction, but it also significantly diminished the amount of Gαq11 in the respective immunoprecipitates (Fig. 4A). Quantification of these data indicates that a two- to threefold increase in the levels of

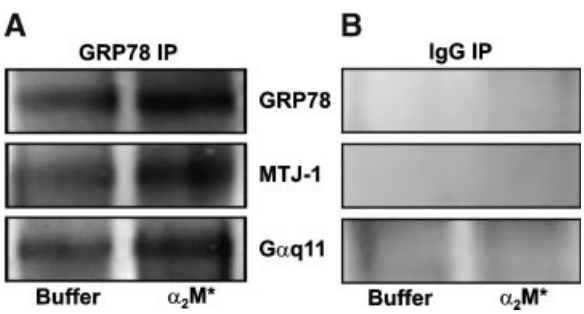

Fig. 1. MTJ-1 and Gαq11 co-immunoprecipitate with GRP78 in plasma membrane lysates from α_2M*-stimulated macrophages. See ''Materials & Methods'' Section for details. **Panel A**: The immunoblots of the GRP78 immunoprecipitates. **Panel B**: Immunoblots performed employing control rabbit IgG in place of specific antibodies. Immunoblots shown are representative of two–three independent experiments. The abbreviation IP is employed to designate immunoprecipitate.

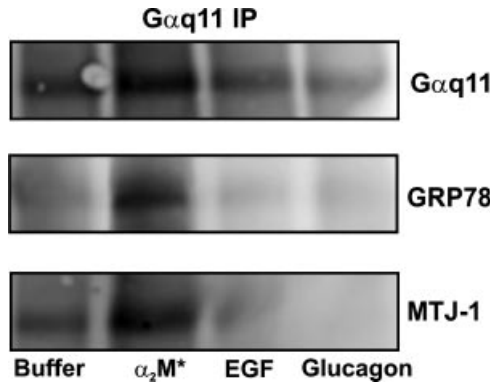

Fig. 2. TJ-1 and GRP78 co-immunoprecipitate with Gαq11 in plasma membrane lysates from α_2M*-stimulated macrophages. See ''Experimental Procedures'' Section for details. Immunoblots shown are representative of two independent experiments. EGF and glucagon were employed as negative controls since neither of their receptors is coupled to Gαq11.

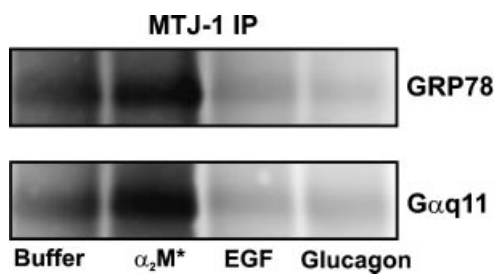

Fig. 3. Immunoblots showing co-immunoprecipitation of Gαq11 and GRP78 with MTJ-1, respectively in plasma membrane lysates from macrophages. Immunoblots shown are representative of two independent experiments.

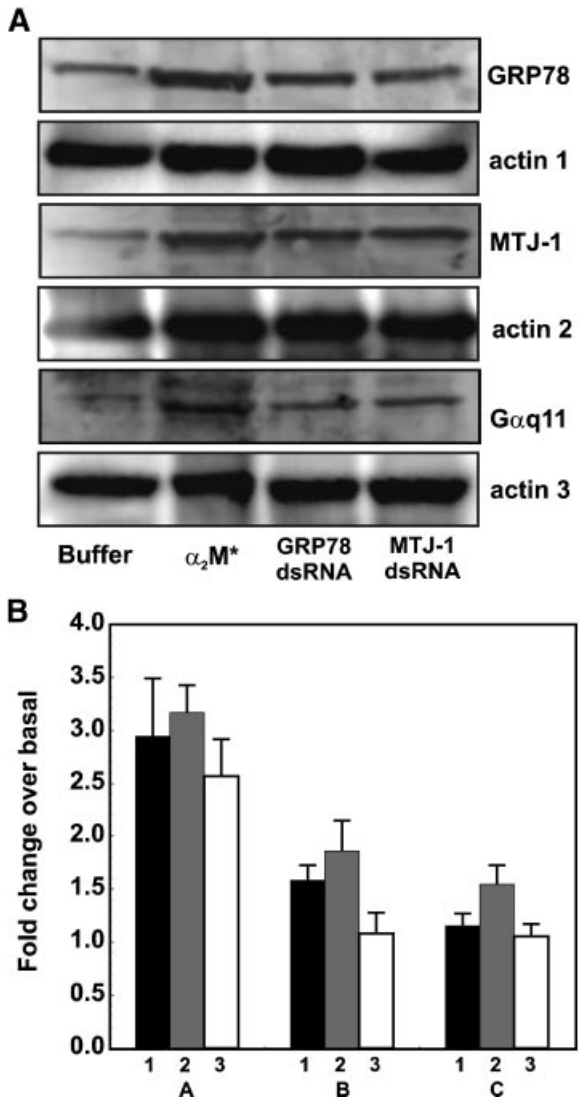

Fig. 4. Effect of silencing the expression of GRP78 or MTJ-1 on the levels of GRP78, MTJ-1, and Gαq11 in GRP78 and MTJ-1 immunoprecipitates of plasma membrane, respectively. Experimental details are described under ''Experimental Procedures'' Section. **Panel A**: The immunoblots shown are representative of two independent experiments. Immunoblots of the protein loading control, actin are shown below GRP78, MTJ-1, and Gαq immunoblots, respectively. **Panel B**: The data from panel A are expressed as fold changes from the buffer-treated cells. The results are expressed as the mean ± SE. The bars are: (1) GRP78, (2) MTJ-1, and (3) Gαq11 in **A**, α_2M*-stimulated cells; **B**, GRP78 dsRNA treated cells prior to α_2M* stimulation; and **C**, MTJ-1 dsRNA-treated cells prior to α_2M* stimulation.

GRP78, MTJ-1, and Gαq11 occurred on stimulation of control cells with α_2M* compared to buffer alone (Fig. 4B). Silencing the expression of GRP78 or MTJ-1 resulted in about a twofold reduction of these proteins with a comparable reduction in Gαq11 in the immunoprecipitates. Moreover, based on the molecular weights of these three proteins, the amounts of GRP78, MTJ-1, and Gαq11 are present in a roughly a 1:1:1 ratio in each of the three immunoprecipitation studies.

Caveolae and Lipid Raft Marker Proteins Caveolin and Flotillin-1, But Not Clathrin, Co-Immunoprecipitate With GRP78 in Plasma Membranes From α_2M*-Stimulated Macrophages

Caveolae/lipid raft microdomains in plasma membranes concentrate G protein-coupled receptors, heterotrimeric G proteins, and G protein-regulated effector molecules to facilitate coordinated and kinetically favorable generation of second messengers [Bickel, 2002; Insel et al., 2005; Stan, 2005; Chatenay-Rivauday et al., 2004]. Several members of the heat shock protein family, including HSP90, 70, 60, and 40 are associated with lipid rafts [Chen et al., 2005]. Heat shock proteins play a role in maintaining the stability of lipid rafts associated signal transduction complexes [Xavier et al., 1998; van der Goot and Harder, 2001; Bickel, 2002; Olsson and Snudler, 2005]. HSP90 associated with Dengue virus receptor complex localizes in lipid rafts [Reyes-Del Valle et al., 2005]. Coxsackie virus A19 infection leads to the accumulation of integrin αvβ3 and GRP78 along with MHC class I molecules in lipid rafts [Triantafilou and Triantafilou, 2003]. Recent studies suggest that the identity of these receptors is GRP78 [Triantafilou and Triantafilou, 2003]. We, therefore, assessed lipid raft association of GRP78 by analyzing the presence of caveolin and flotillin in GRP78 immunoprecipitates of plasma membranes by Western blotting (Fig. 5). Indeed both caveolin and flotillin-1 co-immunoprecipitated with the GRP78 immunoprecipitate in plasma membranes from α_2M*-stimulated macrophages (Fig. 5). However, clathrin a marker of coated pits was not detectable in the GRP78 immunoprecipitate from plasma membranes. Since caveolin and flotillin-1 are specific markers of caveolae and lipid rafts, these results suggest that GRP78 along with Gαq11 and the co-chaperone MTJ-1 all associate with lipid rafts in plasma membranes. Gαq11 proteins are selectively concentrated in caveolae and also interact with the transmembrane scaffolding proteins and various other signaling protein.

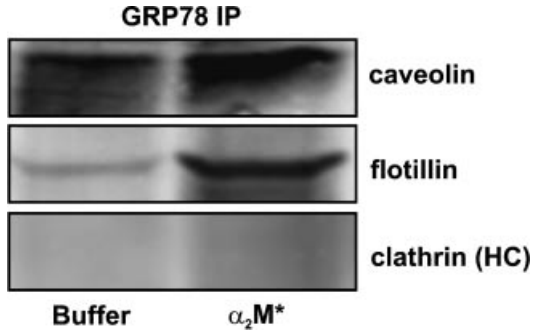

Fig. 5. Co-precipitation of caveolin, flotillin, and clathrin with immunoprecipitated GRP78 in the plasma membrane fraction from α_2M*-stimulated cells. Experimental details are described in ''Experimental Procedures'' Section. A representative immunoblot from duplicate experiments is shown. The protein loading control actin is also shown.

DISCUSSION

Activation of receptor kinases typically occurs by a G protein-dependent increase in the tyrosine phosphorylation of the receptor, leading to recruitment of scaffold proteins via their interaction with phosphorylated tyrosine residues [Guderman et al., 2000; Vazquez-Prdo et al., 2003; Patel, 2004]. This triggers downstream signaling mediated by receptor kinases whose inhibition blocks G protein-dependent signaling [Marinissen and Gutkind, 2001; Patel, 2004]. G proteins can also interact with certain single transmembrane polypeptides [Poppleton et al., 1996; Imamura et al., 1999; Guderman et al., 2000; Hamm, 2001; Marinissen and Gutkind, 2001; Milligan, 2004; Patel, 2004; Sato et al., 2004; Waters et al., 2004; Goren et al., 1985; Vazquez-Prdo et al., 2003]. The PDGF receptor serves as a good model for growth factor receptors. For efficient signal transduction in mammalian cells, the PDGF receptor requires a G protein [Patel, 2004]. Pretreatment with pertussis toxin reduces activation of c-src and p42/44MAPK by PDGF [Patel, 2004]. Two possible signaling pathways from the ligand-bound PDGF receptor have been proposed. One involves a G protein and other involves conventional growth factor receptor autotyrosine phosphorylation followed by recruitment of adaptor/signaling molecules to the receptor. A large body of evidence demonstrates that receptor tyrosine kinases employ proximally located G protein-dependent signaling components in an integrated manner to induce activation of key regulatory pathways linked to cellular proliferation and differentiation [Guderman et al., 2000; Patel, 2004]. Receptor tyrosine kinases in the case of PDGF, insulin, and EGF receptors form complexes with G proteins for use by the receptor tyrosine kinase to promote downstream signaling. Certain kinase receptors like IGF1, directly associate with and activate heterotrimeric G proteins [Marinissen and Gutkind, 2001; Patel, 2004].

GRP78, a molecular chaperone and member of HSP70 heat shock protein family, is expressed constitutively under physiological conditions; however, under ER "stress" induced by a variety of conditions, its synthesis is increased by several fold and it functions to protect the cells from this stress by triggering the unfolded protein response [Kaufman, 1999]. GRP78, like several other KDEL motif containing ER proteins, escapes ER retention and appears on the cell surface [Misra et al., 2002]. In 1-LN prostate cancer cells and murine peritoneal macrophages this cell surface-associated GRP78 functions as a receptor upon binding of proteinase- or amine-activated α_2M* and triggers IP_3/Ca^{2+} signaling, which is not inhibited by pertussis toxin [Misra et al., 1993, 1994a,b, 1995, 1996]. This suggests that α_2M*-induced activation of PI-PLCβ is coupled to the heterotrimeric G protein Gαq11. Recently, we showed that GRP78 is translocated to the cell surface in association with the co-chaperone, MTJ-1, which anchors it to the plasma membrane [Misra et al., 2005a]. GRP78 is not predicated to possess a plasma membrane-spanning domain; however, MTJ-1 is a transmembrane protein which does have a cytoplasmic tail. We, therefore, postulate that the α_2M* signaling receptor is constituted of this binary complex. If this is correct, it should be possible to isolate from plasma membranes a complex of these two proteins and Gαq11. This follows from our previous studies demonstrating that α_2M*-dependent signal transduction requires the participation of a pertussis toxin-insensitive heterotrimeric G protein which is most likely to be Gαq11 [Misra et al., 1994a]. In the present study we demonstrate that MTJ-1 and Gαq11 are co-immunoprecipitated with GRP78 in its plasma membrane immunoprecipitate. We further show that downregulation of either GRP78 or MTJ-1 on the cell surface reduces the presence of Gαq11 in the plasma membrane fraction. These observations explain the loss of α_2M*-dependent cell signaling when either GRP78 or MTJ-1 expression is silenced by RNAi [Misra et al., 2004]. They also for the first time directly demonstrate that

the pertussis toxin-insensitive G protein associated with the α_2M* signaling receptor is Gαq11. GRP78 is a member of heat shock protein family and in several instances GRP78 associated with cell surface, where it binds to α_2M* and elicits several signaling cascades. The results presented also suggest that the binding of α_2M* to GRP78 on cell surface elicits signaling which is mediated via a G protein and a tyrosine phosphorylated receptor. Our data also suggest that GRP78 is associated with lipid rafts/caveolae. These data are of interest for several reasons. Many growth factors and hormones, such as insulin and PDGF, are also located in lipid rafts [Bickel, 2002; Wu and Gonias, 2005] and as noted above, we have postulated that GPR78 functions like a growth factor receptor [Misra et al., 1993, 1994a,b, 1995, 1996, 1997, 1999, 2002, 2004, 2005a; Howard et al., 1996a,b; Misra and Pizzo, 1999, 2002, 1998a,b, 2004; Asplin et al., 2000]. By contrast, the α_2M* receptor LRP is a scavenger receptor which is primarily located in clathrin-coated pits, although a small fraction may transiently associate with lipid rafts [Bickel, 2002].

In conclusion, we show here for the first time that plasma membrane-associated GRP78 forms a ternary signaling complex with MTJ-1 and Gαq11 proteins in the membranes of α_2M*-stimulated cells. The results also confirm our earlier observations, based on use of inhibitors and permeablized cells, that α_2M*-induced signaling is coupled to a pertussis toxin-insensitive G protein [Krieger and Herz, 1994; Misra et al., 1994a,b, 1995, 1996; Misra and Pizzo, 1999, 2002]. These studies were performed prior to the identification of GRP78 as the α_2MSR [Misra et al., 1993, 1994a,b].

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