Dissociation of Insulin Receptor Expression and Signaling from Caveolin-1 Expression*

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The presence of cell surface caveolin/caveolae has been postulated to influence the localization, expression levels, and kinase activity of numerous receptors, including the insulin receptor. However, there are conflicting data concerning the effects of caveolin on insulin receptor expression and function. To help clarify this issue, we created a gain of function situation by expressing caveolin-1 at various levels in HEK-293 cells where the endogenous level of caveolin-1 is very low. We generated four permanent lines of this cell expressing amounts of caveolin-1 ranging from 10 to 40 times that of parental cells. The amount of caveolin-1 in the human embryonic kidney cells expressing the highest caveolin levels is comparable with that of adipocytes, cells that naturally express one of the highest levels of caveolin-1. We measured insulin receptor amount and insulin-dependent receptor autophosphorylation as well as insulin receptor substrate 1 (IRS1) tyrosine phosphorylation as an index of insulin signaling. We found that the insulin receptor level was essentially the same in the parental and all four derived cell lines. Likewise, we determined that insulin-dependent insulin receptor and IRS1 tyrosine phosphorylation was not significantly different in the four cell lines representing parental, low, medium, and high levels of caveolin-1 expression. We conclude that insulin receptor expression and ligand-dependent signaling is independent of caveolin-1 expression.

Caveolae are 50–100 nm invaginations of the plasma membrane that project into the cytosol, and they have been postulated to play numerous important physiological roles in a variety of cell types (1). These postulated caveolae-mediated functions include a role in vesicular transport via endocytosis/transcytosis in the endothelium and other tissues (2–6) and a role as an organizational scaffold for numerous receptors and the components of their signal transduction pathways in a large number of tissues (7). An important biochemical feature of caveolae is that they are enriched in free cholesterol by virtue of the fact that caveolin-1 is a cholesterol-binding protein (8). Caveolae are particularly abundant in adipocytes (9), where they have been postulated to serve as a major site for the initiation of insulin signaling (10). This is a controversial finding, as insulin receptors have been found to be localized in adipocyte caveolae and/or associate with caveolin by some investigators (11, 12) but not others (13, 14). Also, caveolin/caveolae have been suggested to play a role in the regulation of insulin signaling by affecting the phosphorylation of insulin receptor substrate 1 (IRS1) (13) (15). Caveolin-1 knock-out animals have been generated that are viable despite vascular abnormalities and, interestingly, have abnormal adipocytes and develop insulin resistance as they age (16).

Consequently, Lisanti and colleagues have generated adipocytes from caveolin-1 null fibroblasts in order to better understand the phenotype of the caveolin-1 null animals with regard to their insulin responsiveness and the development of insulin resistance (17). They found that such adipocytes had reduced insulin receptor levels compared with those of wild type, and they showed that these levels could be restored to normal levels by transfection of caveolin-1. They also determined that this relationship between insulin receptor levels and caveolin might be a more general phenomenon by showing that transient transfection of caveolin-1 in HEK-293 cells leads to an up-regulation of insulin receptor expression (17). However, in similar experimental protocols other investigators have not observed a relationship between caveolin expression and insulin receptor amounts (15).

Caveolae are thought to be formed by the tissue-specific expression of three caveolin isoforms (caveolin-1, -2, and -3), and they can be induced to form in tissues lacking caveolae by the ectopic expression of caveolin-1, for example (18, 19). The human embryonic kidney cell line, HEK-293, expresses low to negligible levels of caveolin-1 (20). Thus, we sought to help clarify the relationship of insulin receptor expression/function to that of caveolin-1 expression by creating a number of permanent HEK cell lines with levels of caveolin-1 varying from undetectable to approximately that found in adipocytes. We then determined insulin receptor expression levels and ligand-dependent function.

MATERIALS AND METHODS

Antibodies and Western Blotting—Antibodies to caveolin-1, flotilin-1, and phosphotyrosine PY20 were obtained from BD Transduction Laboratories. The anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnologies, Lake Placid, NY. Anti-insulin receptor (21) and anti-IRS1 antibodies were kindly provided by Dr. Jongsoo Lee of the Joslin Diabetes Center, Harvard Medical School. Following SDS-PAGE (22), primary antibodies were detected using appropriate secondary antibodies obtained from Sigma and conjugated to horseradish peroxidase (Sigma) and a chemiluminescence substrate (PerkinElmer Life Sciences). Quantitative analysis of gel bands was performed by scanning blots with the NIH Image 1.63 software program.

Generation of HEK-293 Cell Lines—Mouse caveolin-1a cDNA was produced by reverse transcription PCR from 3T3-L1 adipocytes. The

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1 The abbreviations used are: IRS1, insulin receptor substrate 1; IR, insulin receptor; HEK, human embryonic kidney; PBS, phosphate-buffered saline.
cDNA was then inserted into the pcDNA3.1 Hygro vector and transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Single colonies were selected using Dulbecco's modified Eagle's media (Mediatech Inc., Herndon, VA) containing a mixture of penicillin (5000 units/ml) and streptomycin (5000 μg/ml) (Invitrogen). The media were supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), and 5% calf serum (Invitrogen).

Isolation of Cell Membranes—A postnuclear membrane preparation from the HEK-293 cells was obtained using the method described previously (23). Briefly, cell lines were grown to confluence, scraped into cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 2 mM MgCl2, 1 mM EGTA, and 0.1 mM EDTA) supplemented with protease inhibitors, 1 μM aprotinin, 10 μM leupeptin, 1 μM pepstatin, and 5 mM benzamidine. The lysates were incubated on ice for 30 min and passed through a 21-gauge needle 10 times and centrifuged at 1000 x g to remove nuclear debris. The postnuclear lysate was then centrifuged at 133,000 x g for 30 min. The membrane pellet was resuspended in PBS with the protease inhibitors noted above. An adipocyte plasma membrane was obtained following the enzymatic isolation of epididymal fat pads as we have described (14), following the protocol of Simpson et al. (24).

Cholesterol Determination—The assays were performed as outlined previously (25). Briefly, total and free cholesterol were determined by extracting lipids from HEK cells by the Folch method (26). The lipid phase was hydrolyzed by KOH and ethanol, and a colorimetric assay was used for quantitative analysis.

Immunoprecipitation—Immunoprecipitation was performed for the insulin receptor (IR) (21) and IRS1 (27) as described in the respective references. Cell membranes (IR) or cell lysates (IRS1) were incubated in buffered N Hapes supplemented with 3 μM aprotinin, 10 μM leupeptin, 5 μM pepstatin, 5 mM benzamidine, 100 mM NaF, 2 mM Na3VO4, 20 mM tetrasodium pyrophosphate, 1 mM ammonium molybdate, 5 mM β-glycerophosphate, 30 mM tetrasodium pyrophosphate, and 5 mM EGTA, all adjusted to pH 7.4) containing 0.1% Triton X-100 and an anti-IR or anti-IRS1 antibody for 1 h at 4°C, followed by a 1-h incubation with protein G-Sepharose (Amersham Biosciences) at 4°C. The beads were washed and eluted with 100 μl of 1% Laemmli sample buffer containing 2% SDS and 5% β-mercaptoethanol. Proteins were separated by SDS-PAGE. Blots were probed with a mixture of 4G10 and PY20 or anti-IR and anti-IRS1.

Immunofluorescence—HEK cells were grown in 6-well tissue culture plates containing poly-l-lysine-coated coverslips (BD Biosciences) for 48 h. Cells were then washed one time with PBS and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and then washed two more times with PBS. Cells were blocked and permeabilized by incubation with buffer P (5% donkey serum (Sigma), 5% bovine serum albumin, and 0.5% Triton X-100 in PBS) for 20 min at room temperature. The fixed cells were incubated for 2 h with rabbit anti-caveolin-1 antibody at a dilution of 1:250 in buffer P at room temperature. Four more washes with buffer P were then performed, and the cells were covered in aluminum foil and incubated for 30 min at room temperature with an anti-rabbit Cy3 secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) at a dilution of 1:250. Again, the cells were washed four times with PBS and then mounted with a glycerol/PBS solution (component A of the SlowFade antifade kit from Molecular Probes, Eugene, OR). The stained cells were observed with a Zeiss Axiovert 200M microscope equipped with a Hamamatsu Photonic camera for standard immunofluorescence.

RESULTS AND DISCUSSION

Fig. 1A shows, by Western blotting, the expression levels of caveolin-1, the insulin receptor, and the lipid raft protein flotillin in parental HEK cells and in four cell lines transfected with caveolin-1 cDNA and then selected for different levels of caveolin-1 expression. Caveolin-1 expression varies from 10- to 40-fold higher in these four cell lines than in the parental cells (Fig. 1B), which contain barely detectable levels of caveolin. On the other hand, the insulin receptor levels are essentially the same in all five cell lines, as is the expression of flotillin (Fig. 1, A and B), which was included as a control for the possible effects of caveolin-1 expression on another protein known to be a component of lipid rafts (28).

To determine the localization of expressed caveolin-1, immunofluorescence was performed using an anti-caveolin-1 antibody. Fig. 2 shows the immunofluorescence of HEK cell line B8 expressing a relatively high level of caveolin-1 as compared with that of parental HEK cells. The caveolin-1-expressing HEK cell line, B8, shows expression of caveolin-1 on the surface of cells, whereas the parental HEK cells show no detectable caveolin-1 expression by this technique.

To determine whether there was a functional consequence of caveolin-1 expression in the transfected cells, we measured free cholesterol as shown in Table I. All transfected cells show increased free cholesterol with the two highest expressers expressing a statistically significant 78 and 69% increase, respectively, in free cholesterol as compared with the parental cell line. Because caveolin-1 is a cholesterol-binding protein (8) and free cholesterol is predominantly present at the plasma membrane (29), these results are consistent with expectations and with the caveolin-1 fluorescence data of Fig. 2.

We next determined if the level of caveolin-1 in HEK cells expressing the highest amount of caveolin-1 was comparable with caveolin levels in adipocytes, the cell type in which most studies of the relationship between insulin receptors and caveoli have been performed. Using Western blotting with an anti-caveolin-1 antibody, we compared total membrane preparations from the HEK cells with a rat adipocyte plasma...
membrane preparation. As shown in Fig. 3, caveolin-1 expression in a 25-μg sample from HEK cells is the same as that in a 5-μg sample from rat adipocytes. The purity of the membrane preparation with regard to cell surface markers is >90% for adipocytes (24) and ∼30% for the HEK cells shown. This corresponds to cell surface caveolin-1 levels in the HEK cell line B8 that are at least 60% of that in adipocytes; in other words, a high level of caveolin-1 expression is consistent with its possible biochemical/physiological significance.

We measured receptor autophosphorylation following cellular exposure to insulin in four of the cell lines corresponding to low, medium, and high levels of caveolin-1 expression, as well as in the parental cell. As shown in Fig. 4, following immunoprecipitation of phosphorylated receptor and blotting with an anti-phosphotyrosine antibody, there are no significant differences in receptor autophosphorylation in the cell lines tested and no differences in the overall levels of the insulin receptor (see also Fig. 1). Finally, we measured insulin-dependent phosphorylation of IRS1 to determine whether downstream receptor signaling was affected by caveolin as has been suggested (15). Again we found this not to be the case (Fig. 5), indicating that the cell surface kinase activity of the receptor was not affected by any presumptive interaction with caveolin-1.

The association with and phosphorylation of caveolin-1 by the non-receptor tyrosine kinase, Src, was the basis of the discovery of caveolin-1 as Vip21 (30). Consequently, the putative association of caveolin with and phosphorylation by other non-receptor and receptor tyrosine kinases has been described in many studies (reviewed in Ref. 31). The association of non-receptor tyrosine kinases of the Src family with lipid raft domains, including caveolae, is likely to be a result of their myristoylation, which allows their association with relatively structured lipid raft membrane domains, whereas the transmembrane sequences of receptor tyrosine kinases are likely to be excluded from lipid rafts such as caveolae, as has been predicted by studies of model systems (32). Indeed, in cultured adipocytes, which express robust levels of caveolin-1, the tyrosine phosphorylation of caveolin-1 observed upon activation of the insulin receptor was first described as indirect and resulting from the action of Fyn, a member of the Src kinase family, and not directly by insulin receptors (13). This paper describes cell fractionation protocols to biochemically purify caveolae and reports that the majority of the insulin receptor was excluded from the “floating” fraction of the caveolae enriched in caveolin (13). However, this result was reassessed by some of the same investigators who subsequently reached the opposite conclusion that the interaction of caveolin and the insulin receptor was direct based on their co-fractionation in discontinuous sucrose gradients (12).

Additional biochemical and/or morphological studies in adipocytes have also provided conflicting results favoring or not favoring a direct interaction between caveolin and the insulin receptor. Morphological (electron microscopy) and biochemical analysis of rat adipocytes suggested the co-localization of insulin receptor with caveolae (11). On the other hand, we also used both electron microscopy and an immunoaffinity protocol to rapidly isolate caveolae, and we did not detect any insulin receptor in caveolae by either method (14). We speculate that the reasons for these discrepancies in biochemical analysis most likely lie in the methods used in these various studies, as
we have discussed in detail (14). That is, the time and manipulations required in many protocols of caveolae isolation are factors that are likely to produce artifacts concerning caveolae protein content. The discrepancies in the morphological results are more difficult to explain, although the association of membrane proteins with caveolae can be an artifact of the techniques used (33). Moreover, the observation that transmembrane sequences such as those found in the insulin receptor do not partition into the lipid raft domains characteristic of caveolae (32), and the observation that caveolae are non-dynamic structures (34) in the time frame of the trafficking/endoctyosis of the insulin receptor through clathrin-coated pits (14, 35, 36) makes it seem unlikely to us that adipocyte insulin receptors are localized or associate with caveolae.

Nevertheless, as an independent means addressing the relationship between caveolin-1 and insulin receptors independent of adipocytes, experiments have been performed in cell lines where one can alter the amount of caveolin-1 and determine the functional consequences, if any, of these alterations for signaling from the insulin receptor. In this context, the recent report by Cohen et al. (17) documenting effects of caveolin-1 expression on insulin receptor levels was surprising. These investigators showed that insulin receptor levels are diminished in an adipocyte cell line derived from caveolin-1 null animals but that the receptor levels could be increased by the re-introduction of caveolin-1. We have measured the levels of insulin receptors and caveolin-1 in various adipocyte cell lines such as those described in Refs. 37 and 38, which express these proteins to different extents, and we see no correlation between the two (data not shown). These researchers observed similar results using HEK cells transfected with caveolin-1, albeit they employed transient transfection protocols and described a single result that confirmed their data obtained with caveolin-1 null adipocytes. On the other hand, Yamamoto et al. (15) previously found no effect of caveolin-3 expression on insulin receptor levels or receptor autophosphorylation in transiently transfected HEK cells but did observe a small increase in IRS1 phosphorylation in the transfected cells that they considered significant. Peptides derived from caveolins-1 and -3 were also found to stimulate insulin-dependent IRS1 phosphorylation. These authors (15) attributed these results to a direct interaction of caveolin with the insulin receptor’s kinase domain. However, the receptor sequences they mapped as interacting with caveolin are not obviously accessible to other proteins based on the crystal structure of the kinase domain (39), thus clouding the interpretation of these studies.

Thus, we report here the use of four stable cell lines expressing caveolin-1 at various levels, the highest two approaching that seen in adipocytes. The insulin receptor amount and ligand-dependent activation of its kinase activity, as well as the phosphorylation of its major substrate IRS1, is essentially the same in all of the cell lines and in the non-transfected parental cell. As noted previously, there is no obvious reason why caveolin levels should affect the expression of insulin receptors. These receptors are highly expressed and function perfectly well in the important insulin target cells of the liver where caveolin expression is minimal (40). We conclude that caveolin-1 expression has no effect on insulin receptor expression or function as determined by gain of function experiments in HEK cells, and, for reasons noted above, we believe this is likely to be true for the real insulin target cells of fat and muscle.