Constitutively Active Mitogen-Activated Protein Kinase Kinase Increases GLUT1 Expression and Recruits Both GLUT1 and GLUT4 at the Cell Surface in 3T3-L1 Adipocytes

Yuji Yamamoto, Yasunao Yoshimasa, Makoto Koh, Junko Suga, Hiroaki Masuzaki, Yoshihiro Ogawa, Kiminori Hosoda, Haruo Nishimura, Yoshihiko Watanabe, Gen Inoue, and Kazuwa Nakao

To address a role of mitogen-activated protein kinase (MAPK) in the regulation of glucose transport, we made a constitutively active mutant of MAPK kinase (MAPKK) and introduced it into 3T3-L1 preadipocytes by using a retrovirus-mediated transfection procedure. The deletion of 20 amino acids (those between and including 32 and 51) in the amino terminal region of Xenopus MAPKK and the replacement of serine residues on the 218 and 222 positions by glutamic acid (dSESE-MAPKK) let Xenopus MAPKK constitutively active. The isolated cell clones differently expressing dSESE-MAPKK (clone 219 higher expression, clone 233 lower expression) efficiently differentiated to adipocytes by a standard differentiation cocktail. Accordingly, the increased expression of dSESE-MAPKK protein during differentiation resulted in the increased basal MAPK activity in clone 219 adipocytes and, to a lesser extent, in clone 233 adipocytes. In contrast to clone 233 and parental adipocytes, basal 2-deoxyglucose uptake was enhanced fourfold in clone 219 adipocytes, in accordance with increased expression of GLUT1 mRNA and protein. Whereas GLUT4 mRNA was similarly expressed in all of the adipocytes, GLUT4 protein appeared to decrease in clone 219 adipocytes. More importantly, subcellular fractionation studies showed that the localization of both GLUT1 and GLUT4 in the plasma membranes (PMs) was markedly increased in the basal state in clone 219 adipocytes compared with that in clone 233 and parental adipocytes, in which both glucose transporters were preferentially located in intracellular compartments. Consequently, insulin-induced translocation of GLUT1 was abolished in clone 219 adipocytes, although the remaining intracellular GLUT4 was still responsive to insulin stimulation, which led to the movement to the PM. As combined effects on the situation of GLUT1 and GLUT4, the foldness of insulin stimulation of glucose transport based on the basal activity was reduced in cells expressing constitutively active MAPKK. These results imply that chronic activation of MAPK could be one of the mechanisms for insulin resistance. *Diabetes* 49:332–339, 2000

From the Department of Medicine and Clinical Science (Y.Ya., Y.Yo., M.K., J.S., H.M., Y.O., K.H., H.N., G.I., K.N.), Graduate School of Medicine, and the Department of Molecular Microbiology (Y.W.), Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.

Address correspondence and reprint requests to Yasunao Yoshimasa, MD, PhD, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8397, Japan. E-mail: yayoshi@kuhp.kyoto-u.ac.jp.

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IM, intracellular membrane; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; PI, phosphatidylinositol; PM, plasma membrane; PVDF, polyvinylidene difluoride.

Mitogen-activated protein kinase (MAPK) is activated in response to a wide variety of extracellular stimuli and plays an important role in cell proliferation and differentiation. Activation of MAPK occurs through its dual phosphorylation on threonine and tyrosine residues catalyzed by MAPK kinase (MAPKK), a protein kinase with dual specificity (1). So far, MAPKs are the only specific native substrates for MAPKKs, which are activated by upstream serine-threonine kinases, such as Raf-1, implying that MAPKK is a key intermediate in the MAPK cascade (1).

MAPKKs and MAPKs are widely expressed in various tissues and in terminally differentiated insulin-target organs, such as muscle and fat cells. Insulin increases glucose uptake in the insulin target organs by stimulating the phosphatidylinositol (PI) 3-kinase pathway after binding to its receptor, leading to translocation of insulin-responsive glucose transporter GLUT4 from the intracellular pool to the cell surface (2). On the other hand, the Ras-MAPK pathway does not appear to be involved in the acute insulin action (2). However, it is not fully known whether MAPK plays a significant modulatory role in the glucose transport system. Insulin responsiveness of glucose transport activity is mainly endowed by the characteristics of GLUT4 and GLUT1. GLUT1 is ubiquitously expressed, and the intracellular localization is essentially at the cell surface, whereas GLUT4 is exclusively expressed in the insulin target organs, located predominantly in the intracellular compartments in the basal state, and mediates insulin's stimulatory effect on glucose uptake (3). Therefore, derangement of either an amount or intracellular localization of the glucose transporters could be considered as one of the pathogeneses of the insulin-resistant state.

In this study, we conducted a stable transfection of constitutively active MAPKK into 3T3-L1 cells to address whether constitutive activation of MAPK modulates the glucose transport system. Because MAPKs are the only known substrates of MAPKKs, we could attribute the cause of the changes in intracellular events to the activation of the MAPK.

In addition, because 3T3-L1 adipocytes express both GLUT4 and GLUT1, we were able to examine the two major determinants for the regulation of glucose transport.

RESEARCH DESIGN AND METHODS

Materials and cell lines. Insulin was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Polyclonal anti–ERK-1 and anti–ERK-2 antibodies and anti-GLUT4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–phosphorylated ERK antibody was obtained from New England Biolabs (Beverly, MA). Anti-Xenopus MAPKK antibody against the amino terminal peptide (residues 1–16) was donated by Dr. Eisuke Nishida (Kyoto, Japan) (4). Anti-GLUT1 antibody was kindly donated by Dr. S.W. Cushman (Bethesda, MD).

A plasmid containing the cDNA for constitutively active Xenopus MAPKK (dSESE-MAPKK) was kindly provided by Dr. E. Nishida. The substitution of serine residues at the 218 and 222 positions with glutamic acids and the deletion of the portion in the amino terminal region (from amino acid 32 to 51) of Xenopus MAPKK allowed it to constitutively activate (5). The introduction of the mutant into COS cells markedly activates MAPK (5). Plasmids containing the cDNAs for mouse GLUT1 and rat GLUT4 were kindly donated by Dr. G.I. Bell (University of Chicago, Chicago) and Dr. S. Nagamatsu (Kyorin University, Tokyo), respectively.

To induce dSESE-MAPKK into 3T3-L1 preadipocytes, we used a retrovirus vector (WB-CM) provided by Dr. Y. Watanabe (Kyoto University, Kyoto, Japan), which is derived from Molony murine leukemia virus and has a cytomegalovirus promotor for expression and a neomycin-resistant gene for selection. The dSESE-MAPKK cDNA was inserted into the *Bam*HI site of WB-CM. The vector was transfected into GP+E-86 cells by a calcium phosphate co-precipitation method and cultured with selection medium containing 1 mg/ml of G418 for 5 days and 0.5 mg/ml for an additional 5 days, and colonies resistant to G418 were isolated as previously described (6). The culture media were screened for the titer of a recombinant virus as previously described (6), and the media of the highest virus titer were used for infection to 3T3-L1 preadipocytes.

The 3T3-L1 preadipocytes were seeded in a 6-cm dish and cultured overnight and were cultured subsequently in infectious media containing 10 μg/ml of hexadimethrine bromide (Aldrich Chemical, Milwaukee, WI) for 12 h. Resistant cells were selected with 1 mg/ml G418 after expanding infected cells to four 10-cm dishes. After selection for 10 days, resistant colonies were picked up, expanded, and differentiated to adipocytes to ensure their ability to differentiate by use of isobutylmethylxanthine, dexamethasone, and insulin as previously described (7). Furthermore, the expression of transcript of dSESE-MAPKK mRNA was examined by Northern blot analysis before and after adipocyte differentiation.

Extraction of total RNA and Northern blotting analysis. Total RNA was prepared using TRIzol (Life Technologies, Rockville, MD) according to the manufacturer's protocol. Of total RNA, 9–20 μg was separated by 1.2% agarose-formaldehyde gel electrophoresis and transferred to Biodyne Nylon Membrane (Pall BioSupport, New York). Random-primed ^{32}P probes were generated using cDNAs for dSESE-MAPKK, mouse GLUT1, rat GLUT4, and rat β-actin. After hybridization and washing, the membrane was exposed to the Fuji Imaging Plate and analyzed with the Bio-Imaging Analyzer BAS-2000 (Fuji Photo Film, Tokyo).

Preparation of total cell lysates. Cells were serum-starved for 18 h before all of the experiments. Cells were acutely treated with or without an indicated concentration of insulin, washed twice with ice-cold phosphate-buffered saline, and harvested in a lysis buffer (40 mmol/l HEPES, 10 mmol/l EDTA, 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 1 mmol/l Na_3VO_4, 50 μmol/l okadaic acid, 1% [vol/vol] Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin at pH 7.5). After centrifugation, supernatants were normalized for protein concentration and subjected to immunoprecipitation and immunoblotting.

Preparation of subcellular fractions. When required, cells were treated with 10^{-6} mol/l insulin for 15 min, washed twice with ice-cold phosphate-buffered saline, and immediately homogenized using 20 strokes of a 1-ml Teflon-glass homogenizer in a buffer containing 10 mmol/l Tris-HCl, 1 mmol/l EDTA, 255 mmol/l sucrose, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin at pH 7.4. The homogenized cells were then subjected to subcellular fractionation to isolate plasma membranes (PMs) and intracellular membranes (IMs) as previously described (8). The IM fraction contained both low- and high-density microsomes. The resulting pellets of PMs or IMs were suspended in the homogenization buffer. The protein concentration of both the PMs and IMs was measured by use of the Bradford method.

For immunoblotting, proteins subjected to SDS-PAGE were electroblotted to polyvinylidene difluoride (PVDF) membranes (PolyScreen; NEN Life Science Products, Boston, MA). The filters were blocked with Block Ace (Yukijirushi Nyugyo, Sapporo, Japan) and then incubated with the appropriate antibody, washed, reacted with anti-rabbit or anti-goat IgG coupled to peroxidase, and developed with enhanced chemiluminescence reagents as instructed by the manufacturer (Amersham, Buckinghamshire, U.K.). The signal on the blot was detected and quantified with the Lumino-Image Analyser LAS-1000 System (Fuji Photo Film). With anti-GLUT1 and -GLUT4 antibodies, the signal of GLUT1 and GLUT4 on the blot was proportional to the load over the range that was used.

2-Deoxyglucose transport assay. Glucose transport by monolayers of 3T3-L1 and obtained clone cells was determined by measuring the uptake of 2-deoxy-D-[^{3}H]glucose (100 μmol/l) as previously described (9).

MAPK assay. MAPK activity was measured with an immune complex assay as previously described (10). The incorporation of radioactivity to myelin basic protein was analyzed with the Bio-Imaging Analyzer BAS-2000 (Fuji Photo Film) after separating with SDS-PAGE.

RESULTS

Expression of dSESE Xenopus MAPKK. After selecting clones following retrovirus-mediated transfection of dSESE Xenopus MAPKK (dSESE-MAPKK) cDNA into 3T3-L1 preadipocytes, we examined whether selected clone cells were able to differentiate to adipocytes and whether they expressed dSESE-MAPKK mRNA (Fig. 1*A* and *B*). Among them, we picked two clones (clones 219 and 233) and used them for a further study, because both clones efficiently differentiated to adipocytes with differently expressing dSESE-MAPKK mRNA. The expression of dSESE-MAPKK mRNA by clone 219 cells was ninefold more than that of clone 233 cells. The expression of dSESE-MAPKK mRNA increased around the day 4 postinduction and ultimately by 7.5-fold in mature adipocytes of both clones (Fig. 1*C*). Accordingly, dSESE-MAPKK protein was clearly detected in clone 219 adipocytes, but to a much lesser extent in clone 233, and was not detected in parental adipocytes (Fig. 1*D*).

MAPK activity and adipocyte differentiation. MAPKs are activated by phosphorylation on both threonine and tyrosine residues of the TEY motif by MAPKKs. Therefore, we examined the activation of MAPK by using a specific antibody to recognize only the tyrosine-phosphorylated form of MAPK. As shown in the upper panel of Fig. 2*A*, phosphorylated MAPKs were detected without insulin stimulation in clone 219 adipocytes but much less so in parental and clone 233 adipocytes, whereas endogenous MAPKs (ERK-1 and -2) did not change in their amounts in the parental and clone cells (Fig. 2*A*, lower panel). However, the amount of phosphorylated MAPKs in clone 219 adipocytes was smaller in the basal state compared with that in adipocytes of insulin-stimulated parental and clone cells. Accordingly, basal MAPK activity in clone 219 adipocytes was increased compared with that in basal parental and clone 233 adipocytes but not as much as that in adipocytes of insulin-stimulated parental and clone cells (Fig. 2*B*). However, both clones could be efficiently differentiated to adipocytes by use of a conventional protocol as parental cells (>90%), indicating that such a level of activation of MAPK pathway in 4 days after the differentiation induction did not affect adipogenesis (Fig. 1*B*).

Glucose transport activity. To study the effects of the constitutively active MAPKK on glucose transport activity, we performed 2-deoxyglucose uptake assay using the parental and clone cells before and after adipocyte differentiation. Whereas there were no significant changes before differentiation in the basal and insulin-stimulated glucose transport activity among parental and the two clone preadipocytes, the basal transport activity was significantly increased in clone 219 adipocytes compared with that in parental and clone 233 adipocytes (Fig. 3). Insulin stimulation of clone 219 adipocytes could cause a further increase of glucose transport activity by 80% of

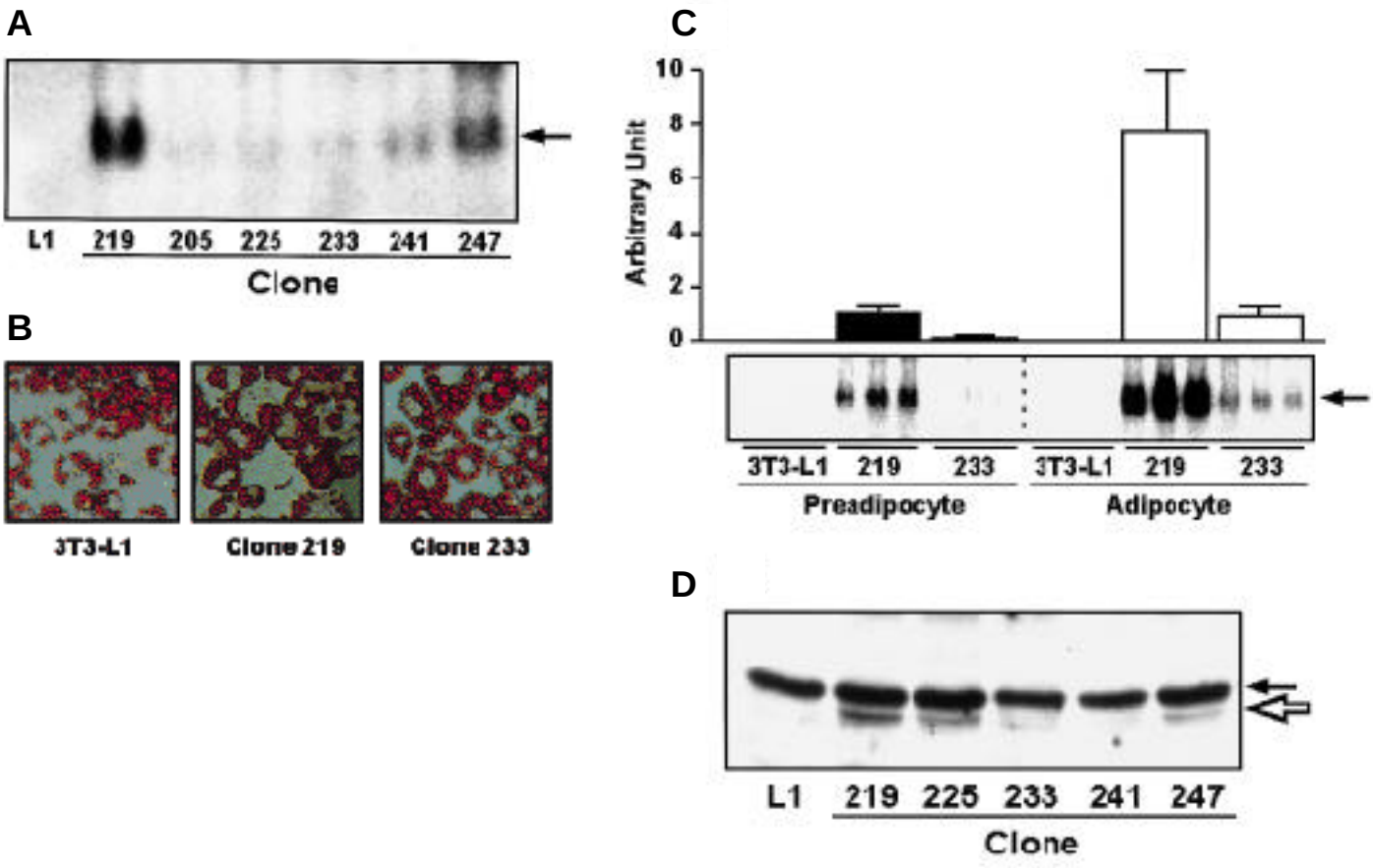

FIG. 1. Isolation of clonal cells expressing constitutively active MAPKK. *A*: The total RNA was prepared from mature adipocytes of isolated clone cells at day 12 postinduction. Expression of Xenopus dSESE-MAPKK mRNA was screened by Northern blot analysis as described in RESEARCH DESIGNANDMETHODS. The corresponding signal (indicated by an arrow) was differently detected in individual clone adipocytes but not in parental 3T3-L1 adipocytes (L1). *B*: Adipocyte differentiation of clones 219 and 233 cells. Fat droplets were stained with Oil Red O at day 12 postinduction. Note that both clones 219 and 233 cells fully differentiated to mature adipocytes, although they differently expressed dSESE-MAPKK (*D*). *C*: The expression of dSESE-MAPKK mRNA before and after differentiation in parental 3T3-L1, clones 219 and 233 cells. Total RNA was obtained from preadipocytes and adipocytes, and it was subjected to Northern blot analysis. The blot is shown in the lower panel and the semiquantified values are plotted in the upper panel. *D*: The expressed dSESE-MAPKK protein was detected by immunoblotting by use of an anti-Xenopus MAPKK antibody. The single arrow indicates endogenous MAPKK, and the double arrows indicate dSESE-MAPKK protein.

the basal value, but the absolute increment of insulin-stimulated glucose transport activity was slightly decreased compared with that in parental and clone 233 adipocytes (Fig. 3). Therefore, it appeared that the insulin-responsive part of glucose transport was not so affected by constitutive activation of MAPK, but the foldness of the insulin stimulation based on the basal activity was markedly reduced.

Glucose transporters. The increased basal glucose transport activity could be rendered by an increased amount and/or increased activity of glucose transporters. The expression of GLUT1 mRNA was not different among parental, clone 219, and clone 233 preadipocytes (Fig. 4*A* and *B*). However, the expression of GLUT1 mRNA greatly increased in clone 219 adipocytes compared with those in parental and clone 233 adipocytes, accompanying a 10-fold increase in GLUT1 protein content (Figs. 4*B*, 5*A*, and 5*C*). On the other hand, GLUT4 mRNA was not detected in preadipocytes, and it increased after adipocyte differentiation in all of the cell lines (Fig. 4*A* and *C*), but the protein level slightly, though significantly, diminished in clone 219 adipocytes compared with that in parental and clone 233 adipocytes (Fig. 5*B* and *D*).

Intracellular distribution of glucose transporters. In addition to the amount of glucose transporters, the intracellular distribution is also important for insulin's regulation of glucose transport. A subcellular fractionation study showed that the basal intracellular distribution of both GLUT1 and GLUT4 was not different between parental and clone 233 adipocytes (Fig. 6). In contrast, in clone 219 adipocytes, the content of both GLUT1 and GLUT4 markedly increased in the PMs (ten- and threefold in GLUT1 and GLUT4 contents, respectively), and GLUT4 reciprocally decreased in IMs in the basal state. Consequently, because the GLUT1 content increased, GLUT1 content in the PM markedly increased in clone 219 adipocytes compared with that in parental and clone 233 adipocytes.

Furthermore, the remaining GLUT1 content in the intracellular compartments of clone 219 adipocytes did not decrease in response to insulin stimulation, whereas GLUT4 content still decreased similarly to that in parental and clone 233 adipocytes (Fig. 6*A* and *B*). Thus, chronic activation of MAPK profoundly affected the intracellular distribution of both GLUT1 and GLUT4.

Taken together, the sustained elevation of MAPK activity increased basal glucose transport activity by increasing GLUT1 expression and causing the increased localization of GLUT1 and GLUT4 at the cell surface. These combined

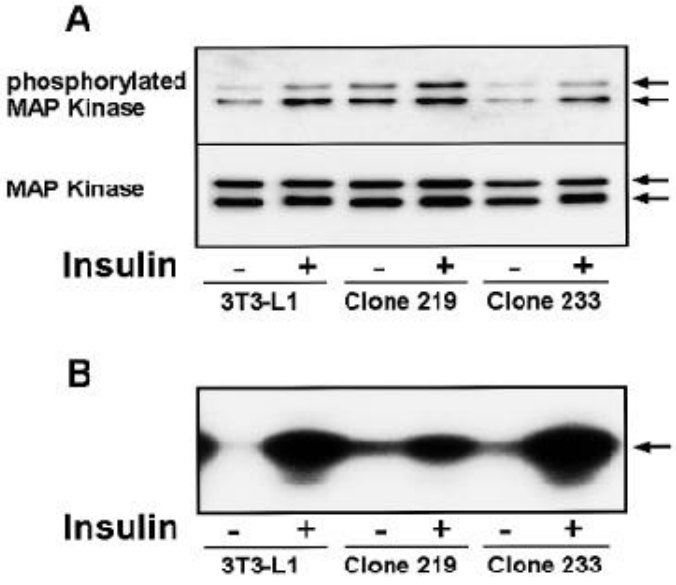

FIG. 2. Phosphorylation and activity of MAPK. Mature adipocytes of parental 3T3-L1 and clone cells were treated with or without 10^{-6} mol/l of insulin for 5 min. Total cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and the phosphorylated MAPK (upper panel) and total MAPKs (lower panel) were detected by semiquantitative immunoblotting (*A*). To examine the activity of MAPK, both ERK1 and 2 were immunoprecipitated from total cell lysates, and the immune complex kinase assay was performed using myelin basic protein as a substrate. The radioactivity incorporated into myelin basic protein (←) was analyzed after SDS-PAGE (*B*).

changes contributed to the diminished insulin-induced increment of glucose uptake by the elevated MAPK activity, although the intracellular GLUT4 was still responsive to insulin stimulation for translocation to the cell surface.

DISCUSSION

In this study, we obtained 3T3-L1 cell clones that differently expressed a constitutively active mutant of Xenopus MAPKK by use of a retrovirus-mediated transfection procedure. Whereas the transfected message was easily detected in both preadipocytes and adipocytes, the expression was increased in adipocytes, resulting in the basal activation of MAPK only in adipocytes. Although the reason why the expression was much less in preadipocytes is unclear, the clones were able to fully differentiate to mature adipocytes. The basal activation of MAPK in the clone adipocytes did not exceed that of fully insulin-stimulated parental cells. These findings clearly indicate that such an endogenous level of MAPK activation in the latter stage does not apparently affect adipogenesis.

In contrast, GLUT1 expression is greatly increased in the adipocytes with sustained elevated MAPK activity, resulting in the increase of basal glucose uptake. Our results are in agreement with previous reports indicating that introducing active mutants of Ras, SHP2, or Raf-1 into 3T3-L1 cells increases the expression of GLUT1 without affecting adipogenesis (11–13). However, Ras and Raf-1 could have substrates and effectors other than MAPKK to transduct the signals downstream (14). Our findings indicate that activation of MAPKK leads to increased expression of GLUT1 without plausible downstream actions of Ras or Raf-1 other than the MAPK kinase (MEK)-MAPK pathway, although we do not completely neglect pathways other than MAPK downstream of MAPKK.

So far, various conditions, including stimulation by growth factors, glucose deprivation, inhibition of protein synthesis, and prolonged low-grade oxidative stress, are known to increase the expression of GLUT1 protein (15–18). The mechanisms underlying the modification of GLUT1 expression could be confined to stimulation of transcription or stabilization of the mRNA. Hypoxia is also shown to increase GLUT1 expression by increasing the transcription via hypoxia-inducible factor 1 (19). Insulin increases GLUT1 expression in primary cultured adipocytes (20) and 3T3-L1 adipocytes (21) while it decreases GLUT4 expression, and it appears to share the pathway for the increased transcription of GLUT1 with the hypoxia-induced pathway (22). On the other hand, some factors stabilize mRNA of GLUT1 by binding to the 3′-untranslated region of GLUT1 mRNA with the mRNA binding protein (23,24), which are reported to be 37- and 40-kDa proteins in the case of the tumor necrosis factor-α effect (25). PED, an overexpressed protein in muscles of type 2 diabetic patients, is also shown to stabilize GLUT1 mRNA, depending on protein kinase C (26). However, it is unknown whether MAPKs are involved in the increase of GLUT1 that is induced by the conditions previously cited. In fact, it is reported that insulin and hypoxia increase GLUT1 expression in L6 cells via p38 MAPK and p70 S6 kinase, but they are not mediated by a MEK-MAPK pathway (27). However, even though neither the hypoxic condition nor insulin increases GLUT1 content by activation of MAPKs, it seems

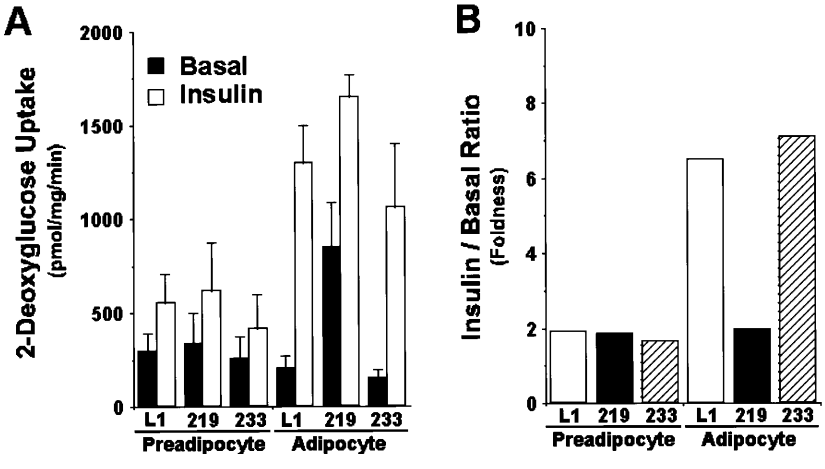

FIG. 3. 2-Deoxyglucose transport activity in 3T3-L1 cells expressing constitutively active MAPKK. *A*: Preadipocytes and adipocytes at day 12 postinduction of parental 3T3-L1 and clones 219 and 233 cells were stimulated with or without 10^{-7} mol/l of insulin for 15 min, and the 2-dexoyglucose uptake was measured as described in RESEARCH DESIGN AND METHODS. *B*: Insulin stimulation of 2-deoxyglucose uptake was estimated by dividing the insulin-stimulated value by the basal value and was then plotted.

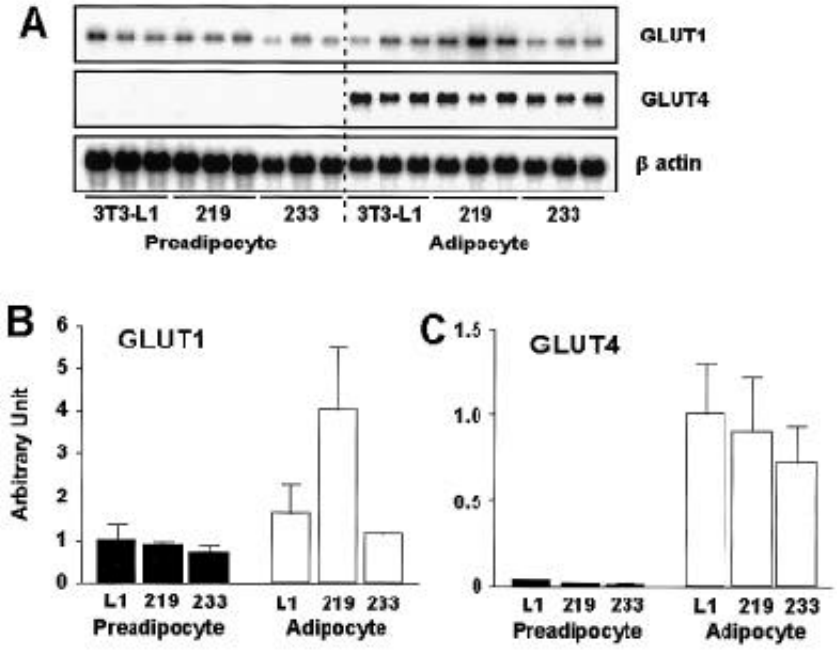

FIG. 4. Expression of GLUT1 and GLUT4 mRNA in 3T3-L1 cells expressing constitutively active MAPKK. Total RNA was prepared from preadipocytes and adipocytes at day 12 postinduction of parental 3T3-L1 and clones 219 and 233 cells and was subjected to Northern blot analysis with probes of GLUT1, GLUT4, and β-actin (*A*). Expression of GLUT1 mRNA (*B*) and GLUT4 mRNA (*C*) was semiquantified and plotted.

certain that the elevation of the Ras-MAPK signaling leads to an increase in GLUT1 expression.

Our data indicate that, in addition to the increased GLUT1 amount, the intracellular distribution of GLUT1 is markedly altered in the adipocytes with elevated MAPK activity, which favors GLUT1 localization at the cell surface. It could be accredited to an "overflow" mechanism that is triggered when the increase of GLUT1 expression exceeds the capacity for its intracellular localization. However, the adipocytes with elevated MAPK activity also have much more GLUT4 at the cell surface with the reciprocal decrease in the intracellular compartments compared with that of parental cells, although the total GLUT4 protein is slightly decreased. Therefore, the alteration in the intracellular localization of GLUT4 cannot be accredited to the level of its expression, and it is likely that a yet undefined phosphorylation of some protein(s) by MAPKK or MAPK is involved in the regulation of intracellular GLUT4 localization. Thus, in addition to the increased GLUT1 content, the increased residency of GLUT4 and GLUT1 in the PMs also appears to contribute to the basal elevation and decreased insulin increment of glucose transport activity, although the relative contribution is difficult to define.

Our finding in regard to the intracellular localization change of GLUT4 by the elevation of MAPK activity is also in agreement with a previous report (28), indicating that activated Ras induces the increased localization of GLUT4 at

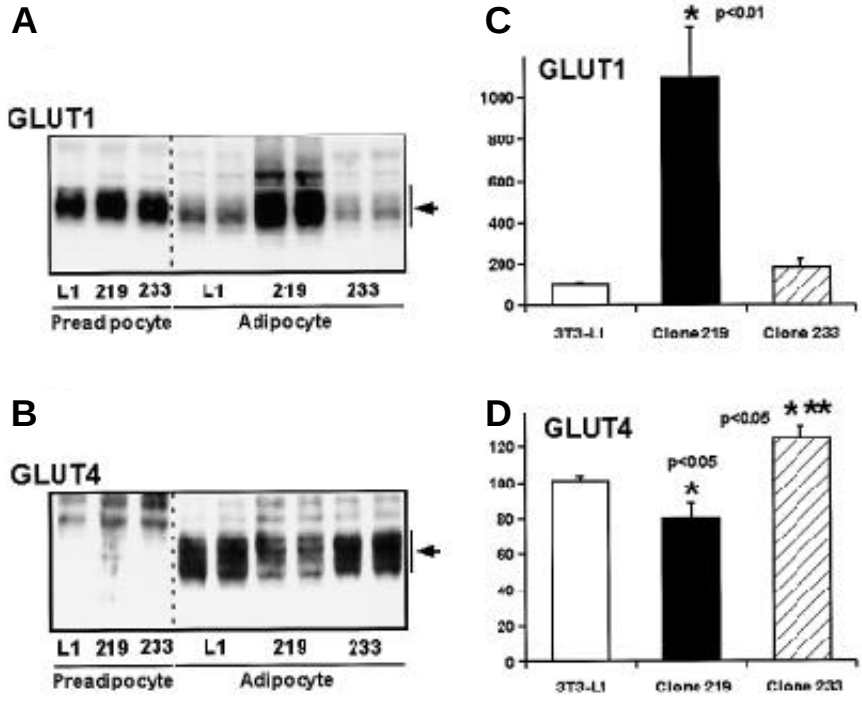

FIG. 5. Amount of GLUT1 and GLUT4 protein in 3T3-L1 cells expressing constitutively active MAPKK. Total cell lysates were prepared from the cells as described in the legend of Fig. 4, and they were subjected to immunoblotting by using anti-GLUT1 (*A*) and anti-GLUT4 (*B*) antibodies. The semiquantified values for the protein contents of GLUT1 and GLUT4 are plotted for GLUT1 (*C*) and GLUT4 (*D*). Total GLUT1 protein content significantly increased in clone 219 adipocytes compared with that in parental and clone 233 adipocytes ($P < 0.01$). Total GLUT4 protein content in clone 219 adipocytes was slightly, though significantly, decreased compared with that in parental and clone 233 adipocytes ($P < 0.05$).

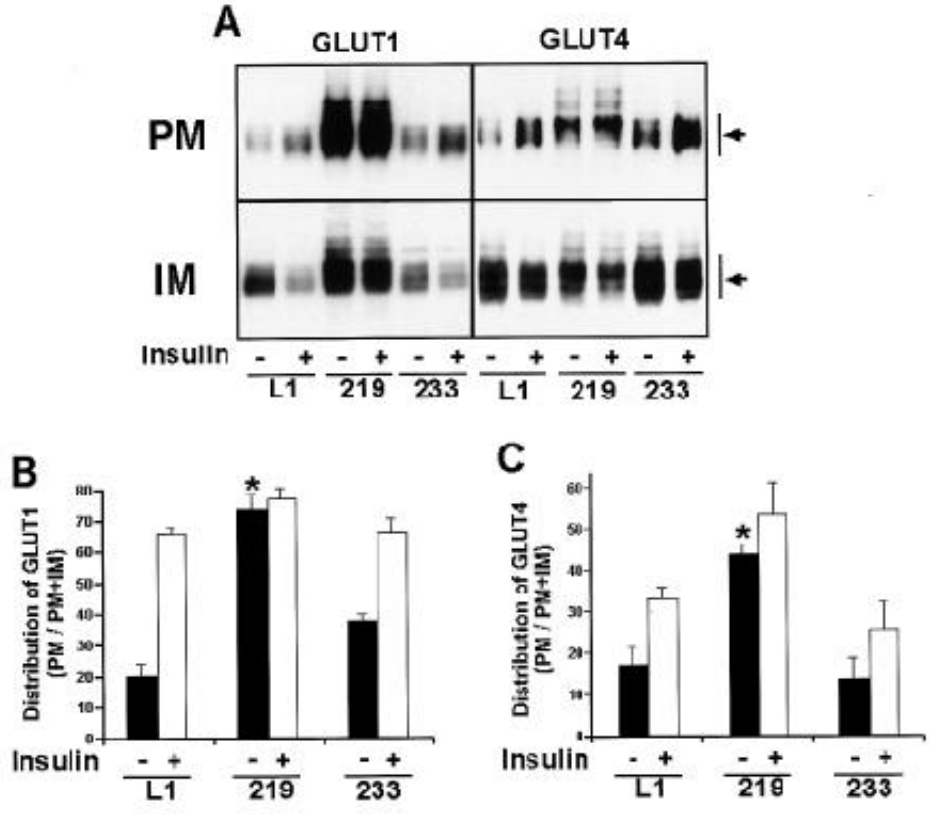

FIG. 6. Intracellular distribution of GLUT1 and GLUT4 in 3T3-L1 cells expressing constitutively active MAPKK. To examine the intracellular distribution of GLUT1 and GLUT4, adipocytes stimulated with or without 10^{-6} mol/l of insulin for 15 min were harvested, homogenized, and subjected to the subcellular fractionation procedure as described in RESEARCH DESIGN AND METHODS. The same protein amount of each fraction was then subjected to SDS-PAGE electrophoresis and immunoblotting with anti-GLUT1 and anti-GLUT4 antibodies (*A*). To evaluate the intracellular distribution of GLUT1 and GLUT4, the recovery of GLUT1 and GLUT4 in each fraction was estimated from the density of the band and the recovered protein amount, and the proportion of their cell surface localization (PM/PM+IM) was calculated as shown for GLUT1 (*B*) and GLUT4 (*C*). Note that the localization in the PMs of both GLUT1 and GLUT4 in the basal state was markedly increased in clone 219 adipocytes compared with those in parental and clone 233 adipocytes. *Significant ($P < 0.05$) differences from basal in parental 3T3-L1 adipocytes.

the cell surface in 3T3-L1 cells. However, they observed a marked reduction of GLUT4 content by the expression of active Ras, which we did not observe by the expression of active MAPKK. Our results are also entirely consistent with reports of the effects of activated Ras that was either transfected into rat adipocytes (29) or microinjected into cardiac myocytes (30). In the former study, cell-surface GLUT4 was increased in the absence of insulin, and in the latter, basal glucose transport increased with no change in GLUT4 gene expression. Finally, isolated fat cells from transgenic mice overexpressing wild-type Ras have increased GLUT1 and GLUT4 at the cell surface in addition to an increased expression of GLUT1 (31). However, a conflicting report concerns the observation in 3T3-L1 cells expressing an active mutant of Raf-1 and describes that the active mutant of Raf-1 does not induce any change in GLUT4 content and its intracellular localization (13). The contradictory results could be ascribed to the difference of the modification sites in the Ras-MAPK pathway. The effectors of Ras, other than the Raf-1– or MEK-mediated pathway, and those of Raf-1, other than the MEK-mediated pathway, might be necessary for the reduction of GLUT4 and the opposing effects on the change of the intracellular localization. Our findings clearly indicate that the sustained elevation of MAPK activity directly induces the preferential localization of GLUT4 and GLUT1 at the cell surface without any other effectors of Ras and Raf-1, although the exact mechanism remains to be elucidated.

The absolute insulin-induced increment of glucose transport activity in cells expressing activated MAPKK was not different from that in parental cells. It is known that GLUT1 is translocated by insulin stimulation as efficiently as GLUT4 in 3T3-L1 adipocytes, although the sequestration of GLUT1 in intracellular compartments is generally not tightly regulated. We show that in the adipocytes with elevated MAPK activity, GLUT1 resides preferentially at the cell surface, and the remaining intracellular GLUT1 cannot respond to insulin stimulation. In contrast, although the intracellular GLUT4 was significantly decreased in the adipocytes with elevated MAPK activity, the remaining GLUT4 in the intracellular compartments could still respond to insulin stimulation to move to the cell surface. These results indicate that elevated MAPK activity does not affect insulin-induced translocation of GLUT4, and that the insulin responsiveness of GLUT4-containing vesicles is preserved, whereas the remaining intracellular GLUT1 seems to be located in vesicles refractory to insulin stimulation.

It is suggested that the increase of GLUT1 content in insulin target organs, such as muscle and fat cells, leads to insulin resistance by decreasing insulin-stimulated glucose transport (32). Mice overexpressing GLUT1 display insulin

resistance, which is not, however, due to glucose-induced resistance of the glucose transport system (33). The increase in GLUT1 content in muscles is also demonstrated in diabetic patients and reported to be negatively correlated with insulin sensitivity (34). However, there is also a report that the overexpression of GLUT1 in skeletal muscle ameliorates insulin resistance (35). It is possible that increased glucose uptake at a whole-body level could ameliorate insulin resistance caused by increased hepatic glucose output, but it would concomitantly further the deterioration of insulin resistance that is caused by the diminishment of insulin's regulatable glucose utilization in muscle and adipose tissues. Further careful analysis is required to determine the different pathophysiological derangement that leads to insulin resistance. On the other hand, many regulations for various metabolic homeostasis and components of various intracellular signaling systems could be modified in insulin-resistant states, and the Ras-MAPK pathway could belong to such a case. In support of this contention, we have observed significantly increased protein levels of Raf-1 and MAPKK in muscles of diabetic obese *ob*/*ob* and KKA^{y} mice, whereas insulin receptors, insulin receptor substrate-1, and several signaling molecules implicated in PI 3-kinase–mediated pathways were considerably decreased as previously described (36 and M.K., G.I., Y.Ya., N. Norisada, M. Shoji, H.M., Y.Yo., K.N., unpublished observations). Therefore, in some diabetic states, it is plausible that MAPK is readily activated by extracellular stimuli other than insulin, which could lead to sustained activation of MAPK in muscles. Such a condition might induce or exaggerate insulin resistance by elevating basal glucose uptake and blunting insulin stimulation, thereby limiting insulin's ability to regulate glucose homeostasis.

In summary, chronic activation of MAPK resulted in the elevated expression of GLUT1 and the increased residency of both GLUT1 and GLUT4 at the cell surface in 3T3-L1 adipocytes. We postulate that the changes in the amount and the intracellular localization of glucose transporters induced by alterations of the Ras-MAPK pathway seriously influence glucose homeostasis and insulin actions in a whole body, leading to the induction or exacerbation of insulin resistance.

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