CONTROL OF ATGL ACTION BY SERINE 517 OF PERILIPIN A GLOBALLY REGULATES PKA-STIMULATED LIPOLYSIS IN ADIPOCYTES

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Running title: Ser 517 of Perilipin A Regulates PKA-Stimulated Lipolysis

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Phosphorylation of the lipid droplet (LD)-associated protein perilipin A (Peri A) mediates the actions of cyclic AMP-dependent protein kinase A (PKA)[§] to stimulate triglyceride (TAG) hydrolysis (lipolysis) in adipocytes. Studies addressing how Peri A PKA sites regulate adipocyte lipolysis have relied on non-adipocyte cell models, which express neither adipose triglyceride lipase (ATGL), the rate-limiting enzyme for TAG catabolism in mice, nor the 'downstream' lipase, hormone sensitive lipase (HSL). ATGL and HSL are robustly expressed by adipocytes that we generated from murine embryonic fibroblasts (MEFs) of perilipin knockout mice. Adenoviral expression of Peri A PKA-site mutants in these cells reveals that mutation of serine 517 alone is sufficient to abrogate 95% of PKA (forskolin)-stimulated fatty acid (FA) and glycerol release. Moreover, a 'phosphomimetic' (aspartic acid) substitution at serine 517 enhances PKA-stimulated FA release over levels obtained with wild type Peri A. Studies with ATGL-and HSL-directed shRNAs demonstrate that 1) ATGL activity is required for all PKA-stimulated FA and glycerol release in MEF adipocytes and 2) all PKA-stimulated FA release in the absence of HSL activity requires serine 517 phosphorylation. These results provide the first demonstration that Peri A regulates ATGL-dependent lipolysis and identify serine 517 as the Peri A PKA site essential for this regulation. The contributions of other PKA sites to PKA-stimulated lipolysis are manifested *only* in the presence of phosphorylated or 'phosphomimetic' serine 517. The primacy of serine 517 in lipolytic regulation is not due to effects on PKA-induced LD dispersion. Thus, serine 517 is a novel 'master regulator' of PKA-stimulated adipocyte lipolysis.

Triglyceride (TAG) and other neutral lipids are stored in adipocyte lipid droplets (LDs) and, in response to energy demand, are hydrolyzed by lipases (lipolysis) to generate fatty acids (FAs) as fuel for peripheral tissues (1-5). Tight regulation of adipocyte lipolysis in response to the inhibitory actions of insulin and the stimulatory actions of lipolytic hormones such as catecholamines maintains whole body energy homeostasis and metabolic health (6-8). Catecholamines bind to β-adrenergic receptors on adipocytes, resulting in upregulation of adenyl cyclase, activation of cAMP-dependent protein kinase A (PKA) and increased lipolytic rate (9). The ability of PKA to stimulate adipocyte lipolysis is mediated in large part by the LD-associated phosphoprotein perilipin (Peri) (1,10,11). Peri A (the predominant perilipin isoform in adipocytes) is the most prevalent PKA substrate in adipocytes. In the absence of hormonal stimulation (i.e., basal state), Peri A functions to sequester lipases from stored neutral lipid, thereby maintaining a low rate of constitutive lipolysis. Following phosphorylation by PKA, Peri A facilitates lipase accessibility to lipid stores, thereby promoting lipolysis (12-20). The mechanism(s) by which Peri A phosphorylation facilitates TAG/lipase interaction in adipocytes is not elucidated.

Previous studies of Peri A function have focused on the ability of Peri A to regulate the lipolytic actions of HSL (15-18). HSL was considered to be the rate limiting lipase for TAG hydrolysis (21-23) until the recent discovery and characterization of ATGL (24-28). Our current view is that the two lipases work hierachically to hydrolyze TAG to FAs and glycerol. ATGL is the 'initiator' lipase, with TAG-selective acylhydrolase activity and limited activity toward DAG. The DAG generated by ATGL action on TAG is then hydrolyzed to (additional) FAs and glycerol by HSL, which is suggested to be more effective as a DAG hydrolase than as a TAG hydrolase (24,26-29). The recent demonstration that ATGL is rate limiting for hormone-stimulated TAG catabolism in adipocytes (28) suggested that the mechanism by which Peri A regulates hormone-stimulated lipolysis must be directed at least in part toward regulating ATGL function. To our knowledge, no study has examined Peri A-dependent regulation of ATGL lipolytic action.

Peri A contains six consensus PKA sites (serine residues) (30) (Fig. 1A). The identification of Peri A PKA site(s) that regulate PKA-stimulated adipocyte lipolysis and the mechanism(s) by which this regulation is achieved has been addressed almost exclusively with non-adipocyte model systems, which lack adipocyte lipases (ATGL and HSL) (15-19). Notwithstanding these limitations, ectopic expression of Peri A PKA site mutants and HSL in these models suggests that PKA-stimulated lipolysis mediated by (ectopic) HSL requires phosphorylation of one or more Peri A N-terminal PKA sites (sites 1, 2 and 3 = serines 81, 223 and 277) (Fig. 1A) (15-17); in contrast, PKA-stimulated lipolysis mediated by (as yet unidentified) endogenous lipase(s) other than HSL requires phosphorylation of one or more Peri A C-terminal PKA sites (sites 4, 5 and 6 = serines 433, 492 and 517) (Fig. 1A) (16). PKA site 5 was recently identified in fibroblasts as a regulator of PKA-induced LD dispersion, an event that is required for chronic (greater than 5h) PKA-stimulated TAG breakdown (19). No function of any other individual Peri A PKA site has been demonstrated.

To obtain a more physiologically realistic picture of how perilipin PKA site phosphorylation regulates adipocyte lipolysis, we developed a stable line of retrovirally-engineered perilipin null (Peri -/-) adipocytes from MEFs of perilipin knockout mice (20). These MEF adipocytes exhibit biochemical and physiological hallmarks of true adipocytes (20). Importantly, these MEF adipocytes robustly express both HSL (20) and ATGL (see Results). Expression of wild-type Peri A fully supports PKA-stimulated lipolysis in these Peri -/- MEF adipocytes, whereas expression of a mutant Peri A lacking all six functional PKA sites (Δ1-6) fails to support PKA-stimulated lipolysis (20). It is unknown whether Peri A mediates lipase-selective regulation of adipocyte lipolysis and if so, how phosphorylation of different Peri A PKA sites hierarchically structures and spatio-temporally coordinates this regulation.

Using adenoviral expression of Peri A PKA site mutants, we now demonstrate that serine 517 (PKA site 6) globally regulates all PKA-stimulated FA and glycerol release in MEF adipocytes. shRNA-mediated 'knockdown' of ATGL and HSL reveals that this regulation reflects the absolute necessity of serine 517 phosphorylation for ATGL-mediated lipolysis in response to PKA activation. These observations provide the first evidence that Peri A regulates ATGL–mediated lipolysis. Moreover, we identify serine 517 as a novel 'master regulator' of PKA-dependent Peri A functions regulating hormone-stimulated lipolysis in adipocytes.

EXPERIMENTAL PROCEDURES

Materials— A specific polyclonal anti-rabbit ATGL antibody was generated using the peptide CTNVAFPPDALRMRAPAS. Antibodies were subsequently affinity-purified and used for Western blotting (1:2000).

Generation and differentiation of stable lines of Peri -/- MEF adipocytes— Stable lines of MEFs were generated from embryos of Peri -/- mice as described (20,31). MEF adipocytes were generated by retroviral expression of PPARγ (31) followed by selection, expansion and differentiation using a standard differentiation medium (20). MEF adipocytes attained a differentiated adipocyte phenotype within 7 days of culturing in differentiation medium (20).

Peri A truncations and PKA site mutants— Adenoviruses expressing the following constructs were generated and verified as previously described (16,20) (Fig. 1A): Aequoria Victoria green fluorescent protein (GFP), Peri A containing serine→alanine mutations at either all six PKA sites (Δ1-6), N-terminal PKA sites (Δ1-3), C-terminal PKA sites (Δ4-6), or individual PKA sites (Δ1, Δ2, Δ3, Δ4, Δ5, and Δ6), and Peri A containing a serine→ aspartic acid mutation at PKA site 6 (Δ6D).

Adenoviral expression of Peri A constructs in Peri -/- MEF adipocytes— Recombinant adenovirus was transduced into Peri -/- MEFs with LipofectAMINE Plus™ (Invitrogen, Carlsbad, CA) on day 2 (adenovirus shRNAs) or day 3 (all other adenovirus) after induction of differentiation. The amount of each adenovirus used was selected to assure comparable levels of expression of the different Peri A constructs, which was confirmed by Western blots and densitometry (16,20).

Recombinant adenoviruses expressing small hairpin RNA (shRNA) directed against murine ATGL or HSL— ATGL shRNA design was based on accession number NM025802 (sequence: GGAGAGAACGTCATCATAT). HSL shRNA design was based on accession number NM010719 (sequence: GCAAGAGTATGTCACGCTA). A 'scrambled' version of these shRNAs (CGCGCTTTGTAGGATTCA) was generated as a control for nonspecific effects of shRNA on lipolysis. All shRNAs were cloned into the pQuiet vector to generate recombinant adenoviruses.

Lipolysis assays— Glycerol and fatty acid release were quantified after 2 h of treatment with/without the PKA activator forskolin (20 μM) as described (15,20).

Quantitative PCR — Total RNA was extracted from Peri -/- MEF cells at days 0, 3, 5 and 7 of differentiation into adipocytes and from 3T3-L1 fibroblasts (16) using a commercial kit (Invitrogen). RNA was quantified by RiboGreen Quantitation Assay (Molecular Probes, Eugene, OR) and cDNA was synthesized from 1 μg of total RNA (Reverse Transcription System, Promega). Real-time PCR was performed in triplicate on an ABI PRISM® 7700 in 20-μl total volume using SYBR® Green PCR Master MIX (Applied Biosystems, Foster City, CA). Primers were designed using Primer Express. Data were analyzed by comparative critical threshold (*Ct*) method and normalized to an endogenous control gene (18 S ribosomal RNA) (32). Percent difference was calculated by $2^{-\Delta\Delta Ct}$.

Immunofluorescence microscopy— After serum depletion, cells were treated for 2 h with either 200 nM PIA to repress adenyl cyclase activity (basal condition) or with 20 μM forskolin (stimulated condition), followed by fixation and incubation with perilipin antibody (16). Images were acquired with a Leica TCS SP2 confocal microscope equipped with an acoustico-optical beam splitter.

Effects of PKA site mutations on forskolin-stimulated LD dispersion— Digital images of a minimum of 200 adipocytes per treatment were obtained from 3-4 separate experiments and were assigned to one of two categories based on Peri A immunoflourescence (19): 1) "clustered," characterized by intact LDs with bright, tight rings of Peri A immunoflourescence and no cytoplasmic Peri A staining, and 2) "dispersed," characterized by partial to total loss of bright rings of Peri A immunoflourescence around large LDs and the appearance of diffuse Peri A staining throughout the cytosol.

Statistical analysis— Data are reported as mean ± SEM. Treatment effects were analyzed by ANOVA using Tukey's procedure for multiple comparisons (Systat v10 for Macintosh, SAS Institute). Significance was set at $P < 0.05$. Percentage data (Table 1) were transformed as arcsin $\sqrt{X}$ before analysis (33).

RESULTS

Phosphorylation of One or More Peri A C-terminal PKA Sites is Required for PKA-stimulated Lipolysis in MEF Adipocytes. Peri A contains six serine residues that are consensus PKA phosphorylation sites (Fig. 1A). We have previously shown that a Peri A construct (Δ1-6)

containing serine to alanine substitutions at all six PKA sites (Fig. 1A) fails to support PKA-stimulated lipolysis in Peri -/- MEF adipocytes (20). In initial studies, we assessed the relative contribution of N-terminal PKA sites (sites 1-3) and C-terminal PKA sites (sites 4-6) to PKA-stimulated lipolysis. We adenovirally transduced Peri -/- MEF adipocytes with wild-type Peri A or with Peri A that contained either 1) serine to alanine substitutions at all PKA sites (Δ1-6), 2) serine to alanine substitutions at the N-terminal PKA sites (Δ1-3), or 3) serine to alanine substitutions at the C-terminal PKA sites (Δ4-6) (Fig. 1B). PKA-stimulated lipolysis (glycerol release) was fully supported by wild-type Peri A and totally abrogated by Δ1-6, confirming our recent observations in MEF adipocytes (20). Notably, total abrogation of PKA-stimulated lipolysis was also observed in Peri -/- MEF adipocytes expressing Δ4-6 (Fig. 1B). This result indicates that one or more Peri A C-terminal PKA sites is required for PKA-stimulated lipolysis in these cells. In contrast, expression of Δ1-3 supported PKA-stimulated lipolysis, although the magnitude of lipolysis was attenuated ~40% (Fig. 1B). This observation indicates that Peri A N-terminal PKA sites are not essential for PKA-stimulated lipolysis in MEF adipocytes; however phosphorylation of one or more N-terminal PKA sites enhances PKA-stimulated lipolysis in the presence of one or more phosphorylated C-terminal PKA sites.

Selective mutation of serine 517 (PKA Site 6) abrogates PKA-stimulated lipolysis in MEF adipocytes. To identify the C-terminal PKA sites that are essential for PKA-stimulated lipolysis, we conducted lipolysis assays with full length Peri A constructs containing individual serine to alanine mutations at PKA sites 4(Δ4), 5(Δ5) or 6(Δ6). Surprisingly, mutation of site 6 alone (Δ6) was sufficient to fully abrogate PKA-stimulated lipolysis (Fig. 2A), thereby recapitulating the inhibitory effects of both Δ1-6 and Δ4-6 (Fig. 1B). Thus, functional PKA sites 1-5 are insufficient to promote *any* PKA-stimulated increase in lipolysis in the absence of serine 517 phosphorylation. This result indicates that serine 517 is essential for PKA-stimulated lipolysis.

Adenoviral expression of Δ5 containing a mutant serine 492 (PKA site 5) resulted in partial (~30%) inhibition of PKA-stimulated lipolysis (Fig. 2A), indicating that in the presence of phosphorylated serine 517 (and PKA sites 1-3), serine 492 enhances PKA-stimulated lipolysis. In contrast, mutation of PKA site 4 had no effect on PKA-stimulated lipolysis (Fig. 2A).

Adenoviral expression of full length Peri A constructs containing serine to alanine substitutions at individual N-terminal PKA sites 1(Δ1), 2(Δ2) or 3(Δ3) failed to diminish PKA-stimulated lipolysis as compared with wild type Peri A (Fig. 2B). As mutation of all three N-terminal PKA sites (Δ1-3) partially attenuated PKA-stimulated lipolysis (Fig. 1B), these results indicate that Peri A N-terminal PKA sites enhance PKA-stimulated lipolysis by a mechanism requiring phosphorylation of at least two of the three sites. As observed for PKA site 5, this mechanism promotes PKA-stimulated lipolysis only in the presence of phosphorylated serine 517 (Fig. 2A).

Serine 517 (PKA Site 6) Regulates PKA-stimulated Lipolytic Actions of ATGL. Consistent with their differentiated adipocyte phenotype, Peri -/- MEF adipocytes robustly express both HSL (20) and ATGL (Fig. 3A), the rate-limiting lipase for TAG catabolism in adipocytes (28). To determine the relative contribution of ATGL to PKA-stimulated lipolysis in MEF adipocytes, we measured PKA-stimulated FA and glycerol release from MEF adipocytes in the presence and absence of ATGL–directed shRNA (Fig. 3B). ATGL-directed shRNA reduced ATGL protein expression by ~100%, and this reduction was coincident with total abrogation of forskolin-induced FA release (Fig. 3B, upper panel). Note that a small increment of PKA-stimulated FA release is Peri A-independent (observed in GFP-expressing cells), and that this Peri A-independent lipolysis is also blocked by ATGL-directed shRNA. In addition to its inhibitory effect on FA release, ATGL-directed shRNA completely blocked forskolin-induced glycerol release (Fig. 3B, lower panel). This result is consistent with the view that ATGL is the 'initiator' TAG lipase upstream of HSL and that HSL acts exclusively on DAG in MEF adipocytes (24,26). Thus, ATGL lipolytic activity is required for essentially all lipolysis in MEF adipocytes. This predominant role of ATGL in PKA-stimulated lipolysis strongly suggested that

mutation of serine 517 of Peri A abrogates PKA-stimulated lipolysis (Fig. 2A) by blocking ATGL lipolytic action at the LD surface.

To test the role of serine 517 in ATGL-mediated lipolysis, we conducted additional lipolysis assays in MEF adipocytes that expressed HSL-directed shRNA. We reasoned that in the absence of HSL, residual lipolytic activity due to ATGL would generate FAs (but not glycerol) and would be completely blocked in cells expressing mutant serine 517. Expression of shRNA directed against HSL resulted in ~95% abrogation of both HSL expression (Fig. 3C) and HSL activity (i.e., glycerol release) (data not shown; see also (20)). However, PKA-stimulated FA release was attenuated by only ~70% relative to cells expressing GFP (Fig. 3C). The ~30% residual FA release in cells expressing HSL-directed shRNA is consistent with the proposed role of ATGL as a TAG-selective acylhydrolase with little or no activity toward DAG (24,26,28). Importantly, all of this residual non-HSL-mediated FA release in response to PKA activation was blocked in cells expressing mutant serine 517 (Δ6) (Fig. 3C). These results demonstrate that phosphorylation of serine 517 is required for ATGL-mediated adipocyte lipolysis in response to PKA.

To assess whether phosphorylation of serine 517 is sufficient for PKA-stimulated lipolysis, we generated a phosphomimetic charge mutant by substituting aspartic acid (D) for serine 517 (Δ6D). We reasoned that if phosphorylation of serine 517 was sufficient to promote PKA-stimulated lipolysis, expression of Peri AΔ6D in Peri -/- MEF adipocytes would stimulate lipolysis in the absence of PKA activation. Expression of Δ6D in Peri -/- MEF adipocytes resulted in a significant (35%) enhancement of PKA-stimulated FA release relative to wild type Peri A ($P < 0.05$; Fig. 4). However, expression of Δ6D had no detectable effect on basal state release of fatty acids (Fig. 4) or glycerol (data not shown). Thus, the presence of an acidic residue at position 517 of Peri A results in a significantly enhanced lipolytic response to PKA but does not increase lipolysis in the absence of PKA activation.

Effects of Peri A PKA Site Mutants on Lipid Droplet Dispersion. PKA activation induces the fragmentation of LDs into small Peri A-coated microdroplets, which disperse throughout the cytoplasm. This LD dispersion is implicated in the regulation of PKA-stimulated lipolysis (19,34-36). Although serine 492 (PKA site 5) is reported to regulate PKA-induced LD dispersion, the role of serine 517 phosphorylation in this process has not been addressed (19). Accordingly, we asked if the requirement of serine 517 phosphorylation for PKA-stimulated lipolysis reflected an essential role for serine 517 in LD dispersion. Using Peri A immunofluorescence and confocal microscopy, we graded forskolin-induced (2h) LD dispersion in MEF adipocytes expressing either wild type Peri A or the PKA site mutants Δ6, Δ6D, and Δ5 (see Experimental Procedures). LDs were graded as either clustered (no dispersion) or dispersed (either partially or fully). These data are summarized in Table 1, with representative confocal images presented in Fig. 5.

Forskolin treatment of MEF adipocytes expressing wild type Peri A resulted in 92% of cells with either partially or fully-dispersed LDs (Table 1; Fig. 5, panel B). Forskolin treatment of cells expressing Δ6 resulted in a frequency of LD dispersion (86%) that was not significantly different from the frequency obtained with wild type Peri A ($P > 0.05$) (Table 1; Fig. 5 panel D). Moreover, in the absence of PKA activation by forskolin, the serine 517 phosphomimetic (Δ6D) failed to induce LD dispersion over levels observed in MEF adipocytes expressing wild type Peri A (data not shown). These results argue that serine 517 phosphorylation does not contribute to PKA-induced LD dispersion in MEF adipocytes. In contrast, expression of Δ5 significantly (~37%) attenuated PKA-induced LD dispersion relative to Peri A ($P < 0.03$) (Table 1; Fig. 5 panel F). This result confirms a role for serine 492 phosphorylation in PKA-induced LD dispersion (19).

DISCUSSION

Peri A is the 'gatekeeper' of adipocyte TAG storage and hydrolysis. Consistent with this physiological function, Peri A polymorphisms and protein levels are associated with risk for obesity and insulin resistance (37,38). How Peri A regulates TAG storage and hydrolysis in adipocytes remains controversial (20,39). The present study exploits a novel adipocyte model

system derived from Peri -/- MEFs to investigate how phosphorylation of Peri A PKA sites regulate hormone-stimulated lipolysis in adipocytes. As in murine adipocytes *in vivo* (24,28), Peri A -/- MEF adipocytes express both ATGL and HSL, with PKA-dependent TAG hydrolysis initiated by and dependent upon ATGL (Figs 3B,C). This 'initiator' function of ATGL renders it rate-controlling for lipolysis in mouse adipocytes (27,28). Here, we demonstrate for the first time that the ability of Peri A to globally regulate PKA-stimulated lipolysis in adipocytes reflects Peri A-dependent regulation of ATGL lipolytic function. Moreover, we identify phosphorylation of serine 517 of Peri A as essential for ATGL-mediated (and thus HSL-mediated) adipocyte lipolysis in response to PKA activation. Mutation of serine 517 to alanine is sufficient to completely abrogate PKA-stimulated FA and glycerol release to levels observed in the absence of Peri A (Fig. 2A) or in the presence of PKA site-deficient Peri A (Δ1-6, Fig. 1B). These data are the first to identify a role for serine 517 in lipolysis and provide the first demonstration that adipocyte lipolysis can be acutely and globally regulated by a single Peri A amino acid.

To address the mechanism of serine 517 action, we asked if phosphorylation of serine 517 was required for PKA-stimulated LD dispersion, an event implicated in PKA-stimulated lipolysis (19,34-36). The almost negligible effect of serine 517 mutation on PKA-stimulated LD dispersion (Table 1) strongly suggests that serine 517 regulates ATGL-mediated lipolysis by a mechanism other than LD dispersion. However, our data do not rule out the possibility that serine 517 phosphorylation alters LD structure on a scale below the resolution of the studies reported here and that this alteration in LD structure is required for ATGL-mediated lipolysis. With regard to mechanism, Peri A is currently viewed as a component of a dynamic scaffold that serves as an LD-associated organizing center for enzymes and transporters involved in lipid metabolism (40). Metabolically-regulated changes in the conformation and composition of this scaffold are proposed to inhibit or promote lipolysis. In this context, serine 517 phosphorylation may function as an electrostatic or conformational 'switch' that regulates the selective interaction of ATGL and/or modulators of ATGL function (27) with LDs. Elucidation of how serine 517 phosphorylation alters the LD 'interactome' to facilitate ATGL-mediated lipolysis will provide critical insight into the molecular mechanism(s) of TAG storage and hormone-stimulated lipolysis in adipocytes.

Although phosphorylation of serine 517 was determined to be the rate-controlling event in PKA-stimulated lipolysis in MEF adipocytes, mimicking this event by expression of a serine 517 charge mutant (Δ6D) was not sufficient to induce lipolysis in the absence of PKA activation (Fig. 4). This result may reflect the fact that phosphorylation of serine 517 facilitates lipolysis by a non-electrostatic mechanism that is not 'mimicked' by the charge mutant (19). Alternatively, phosphorylation of other intracellular PKA targets may be required for ATGL-mediated lipolysis in response to PKA activation. One likely target is serine 492 (site 5), which regulates PKA-stimulated LD dispersion and TAG turnover in fibroblasts (19). In the present study, mutation of serine 492 partially attenuated both LD dispersion (Table 1) and PKA-stimulated lipolysis (Fig. 2A), consistent with a role for LD dispersion in PKA-stimulated lipolysis in MEF adipocytes. As in 3T3-L1 adipocytes (24), the preponderance of ATGL is dispersed throughout the cytosol in MEF adipocytes (data not shown). This localization suggests a model in which serine 492-mediated LD dispersion facilitates lipolysis by enhancing TAG interaction with cytosolic ATGL. However, as clearly demonstrated in the present study, full LD dispersion is insufficient to promote any TAG hydrolysis in the absence of phosphorylated serine 517 (Fig. 5 panel D).

In the present study, mutation of PKA sites 1-3 partially blocked PKA-stimulated glycerol release, and, thus HSL action, consistent with prior studies demonstrating a role for sites 1-3 in promoting HSL-mediated lipolysis (15-18). We demonstrate for the first time that, in contrast to PKA sites 6 and 5, mutation of individual PKA sites (1, 2, or 3) has no effect on lipolysis (Fig. 2B), indicating that Peri A N-terminal PKA sites modulate HSL-mediated lipolysis via a multi-site mechanism. This mechanism is not required for PKA-induced LD dispersion in MEF adipocytes (data not shown) or in fibroblasts (19), but it may promote tight binding of HSL with the LD surface (20). Irrespective of the exact mechanism by

which PKA sites 1-3 promote HSL-mediated lipolysis, the mechanism is insufficient to promote any PKA-stimulated lipolysis in the absence of serine 517 phosphorylation. This may in part reflect the unavailability of DAG (the preferred HSL substrate) in the absence of ATGL-dependent TAG hydrolysis. The involvement of Peri A PKA sites 1-3 and 5 in hormone-stimulated lipolysis raised the possibility that serine 517 is required for phosphorylation of these sites. However, mutation of serine 517 to alanine does not inhibit PKA-dependent phosphorylation of these other PKA sites (1-5) (Supplemental Fig. 1). Elucidation of the molecular mechanism underlying lipolytic regulation by serine 517 remains an exciting challenge for future studies.

Considered together, these observations support the designation of serine 517 of Peri A as a 'master regulator' of PKA-dependent lipolysis and identify ATGL as a critical target of serine 517 action. The primacy of serine 517-dependent regulation among the multiple lipolytic regulatory mechanisms in MEF adipocytes reflects the function of ATGL as the 'initiator' lipase in TAG hydrolysis. A similar function for ATGL is reported for mouse adipocytes (28), suggesting that serine 517 globally regulates PKA-stimulated lipolysis *in vivo*.

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FOOTNOTES.

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§Abreviations used are: ATGL, adipose triglyceride lipase; cAMP, cyclic AMP; DAG, diglyceride, FA, fatty acid; GFP, green fluorescent protein; HSL, hormone sensitive lipase; LD, lipid droplet; MEF, murine embryonic fibroblast; TAG, triglyceride; Peri A, perilipin A; Δ1-6, Peri A mutant containing serine to alanine mutations at PKA sites 1-6; Δ1-3, Peri A mutant containing serine to alanine mutations at PKA sites 1, 2 and 3; Δ4-6, Peri A mutant containing serine to alanine mutations at PKA sites 4 ,5 and 6; Δ1, Δ2, Δ3, Δ4, Δ5, Δ6; Peri A mutants containing an individual serine to alanine mutation at PKA site 1, 2, 3, 4, 5 or 6; Peri AΔ6D, Peri A mutant containing an aspartic acid substitution for serine at PKA site 6.

FIGURE LEGENDS

Fig. 1. **Phosphorylation of one or more Peri A C-terminal PKA sites is essential for PKA-stimulated lipolysis in MEF adipocytes.** *(A)*, Diagram of Peri A and Peri A truncations, indicating the relative position of serine residues(s) of the six consensus PKA phosphorylation sites. *(B)*, PKA-stimulated lipolysis (glycerol release) is abrogated by serine→ alanine mutation of Peri A PKA sites 1-6 (Δ1-6) and 4-6 (Δ4-6), and partially blocked by mutation of PKA sites 1-3 (Δ1-3). Peri -/- MEF cells were transduced with adenovirus expressing GFP (negative control), Peri A, or mutated Peri A. After differentiation to adipocytes, cells were depleted of serum and treated for 2 h in the absence (-, open bars) or presence (+, shaded bars) of the PKA activator forskolin (20 μM). The medium was collected and glycerol content was determined. Data (mean ± SEM) are from 3-7 experiments performed in at least duplicate and are normalized to PKA-stimulated lipolysis in cells expressing Peri A. Lower panel: Western blot with anti-perilipin IgG (15) showing comparable levels of adenovirally-expressed proteins in a representative experiment. **, $P < 0.01$.

Fig. 2. Phosphorylation of serine 517 is essential for PKA-stimulated glycerol release. *(A*), Upper panel: PKA-stimulated lipolysis is fully supported by Peri A containing a mutant PKA site 4 (Δ4), partially inhibited by a Peri A containing mutant PKA site 5 (Δ5) and completely inhibited by Peri A containing a mutant PKA site 6 (Δ6). Lipolysis assays (2 h) were conducted in the absence (-, open bars) or presence (+, shaded bars) of the PKA activator forskolin (20 μM). Data are from 3 experiments performed in at least duplicate. Lower panel: Representative Western blot with anti-perilipin IgG (15). *(B),* Individual N-terminal PKA site mutants (Δ1, Δ2 or Δ3) fully support PKA-stimulated lipolysis. Data are from 5 experiments performed in at least duplicate. Lower panel: Representative Western blot with anti-perilipin IgG (15). **, $P < 0.01$.

Fig. 3. **Serine 517 globally regulates PKA-stimulated lipolysis by regulating ATGL.** *(A),* ATGL gene expression is upregulated ~85-fold during the differentiation of Peri -/- MEF adipocytes. mRNA levels

(ATGL / 18 S) were quantitated by SYBR Green real-time PCR and expressed relative to levels measured at day 7. Note negligible ATGL expression in 3T3-L1 fibroblasts. Lower panel: Representative Western blot with anti-ATGL IgG. *(B),* shRNA knockdown of ATGL abrogates all PKA-stimulated release of FAs (upper panel) and glycerol (lower panel). Peri -/- MEFs were transduced with adenovirus (Ad) expressing either GFP (control), or Peri A in conjunction with either ATGL-directed shRNA or 'scrambled' shRNA (see Experimental Procedures). Results are expressed as mean ± SEM of 4 experiments performed in at least duplicate. Representative Western blots with anti-ATGL IgG and anti-perilipin IgG (15). **, $P < 0.01$. (*C)* Residual lipase activity following shRNA knockdown of HSL is fully blocked by mutation of serine 517 (Δ6). MEF adipocytes received either HSL-directed shRNA or 'scrambled' shRNA. Lower panel: Representative Western blots with anti-HSL IgG (41) and anti-perilipin IgG. **, $P < 0.01$.

Fig. 4. Δ6D, a phosphomimetic (serine→aspartic acid) substitution at serine 517, does not constitutively elevate basal lipolysis but does promote enhanced PKA-stimulated lipolysis over that obtained with wild-type Peri A. Lipolysis assays were conducted as in Fig. 1. Results are expressed as mean ± SEM of 3 experiments performed in at least duplicate. Lower panel: Representative Western blots with anti-perilipin IgG. **, $P < 0.01$.

Fig. 5. Serine 517 regulates PKA-stimulated lipolysis independently of LD dispersion. Peri -/- MEFs were transduced with adenovirus and differentiated to adipocytes. After serum depletion, MEF adipocytes were treated for 2 h with either 200 nM PIA (basal condition) or with 20 μM forskolin (stimulated condition), followed by fixation and incubation with anti-perilipin IgG (see Experimental Procedures). (*A,B*), In cells expressing Peri A, LDs are aggregated under basal conditions, but are disrupted into microdroplets that are dispersed throughout the cell in response to PKA activation. (*C,D*), A similar pattern of PKA-induced LD disruption is predominant among cells expressing the serine 517 mutant, Δ6 (see also Table 1). (*E,F*) In contrast, ~40% of cells expressing the serine 492 mutant, Δ5 exhibit aggregated LDs in response to forskolin. *Scale bar* = 10 microns.

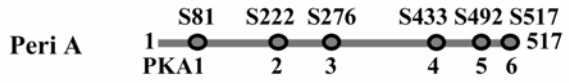
Table 1. Effect of Peri A PKA site mutants on PKA-induced LD dispersion. MEF adipocytes (N) were treated for 2 h with forskolin and analyzed for LD dispersion by Peri A immunoflourescence and confocal microscopy (see Experimental Procedures). Data from 3-4 separate experiments are summarized below and were analyzed by ANOVA following transformation (see Experimental Procedures). Numbers in parentheses are the proportion of cells with LDs designated as partially-dispersed and fully dispersed, respectively (19).

Perilipin	N	% Cells With Aggregated LDs	% Cells With Dispersed LDs
Peri A	255	7.8	92.2 (14.5, 77.6)
Δ6	228	14.0 ns	86.0 (31.6, 54.4)
Δ5	283	37.6**; *	62.4 (26.6, 35.8)

ns, not significantly different from Peri A, $P > 0.05$
**, significantly greater than Peri A, $P < 0.01$
*, significantly greater than Δ6, $P = 0.05$

Fig. 1

A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S222
S276
S433
S492
S517
Peri A
1
517
PKA1
2
3
4
5
6

B

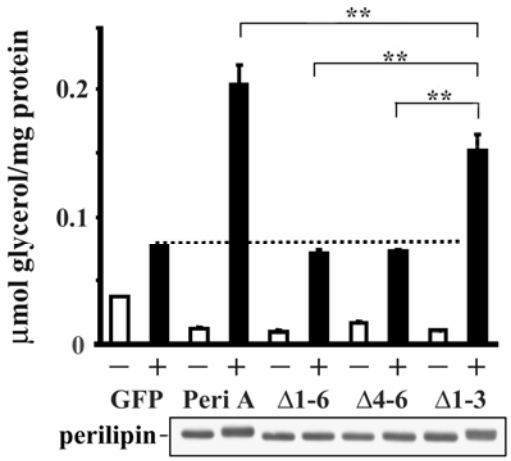

**
**
**
µmol glycerol/mg protein
0.2
0.1
0
− + − + − + − + − +
GFP
Peri A
Δ1-6
Δ4-6
Δ1-3
perilipin

Fig. 2

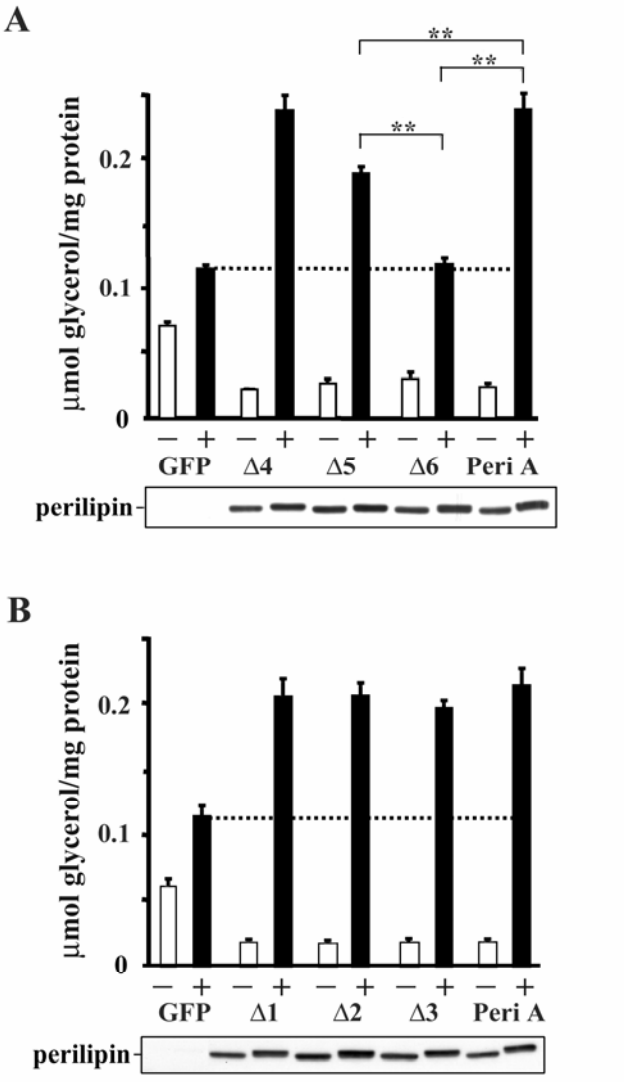
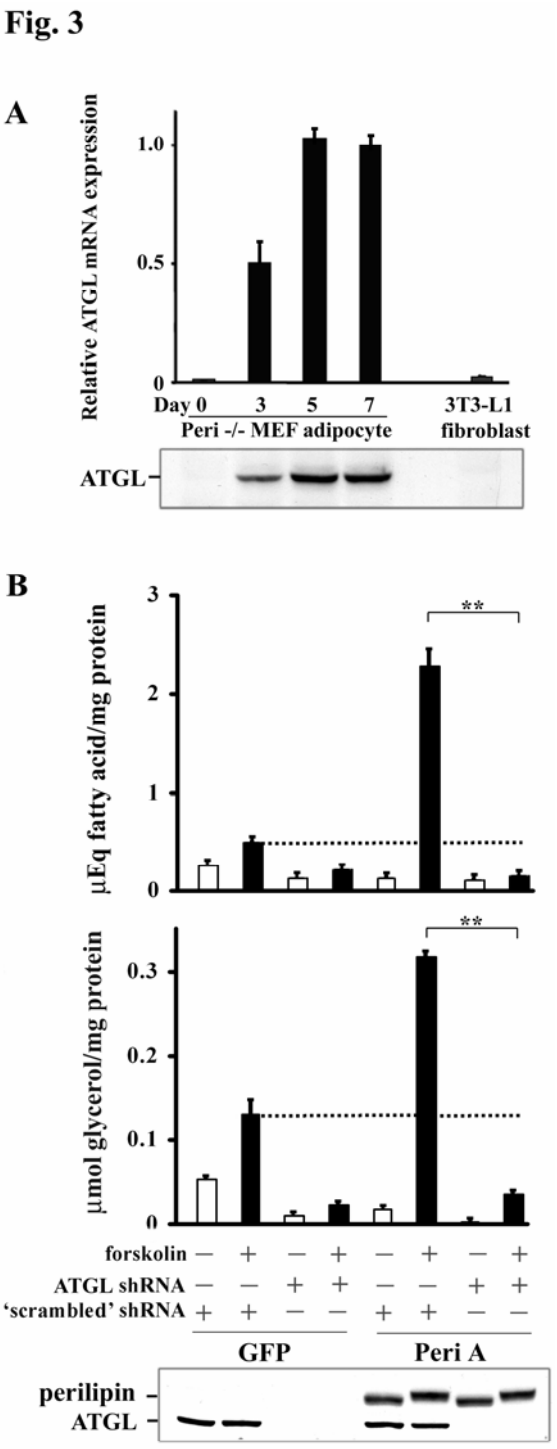

A
**
**
**
μmol glycerol/mg protein
0.2
0.1
0
− + − + − + − + − +
GFP Δ4 Δ5 Δ6 Peri A
perilipin
B
μmol glycerol/mg protein
0.2
0.1
0
− + − + − + − + − +
GFP Δ1 Δ2 Δ3 Peri A
perilipin

Fig. 3


A
Relative ATGL mRNA expression
1.0
0.5
0
Day 0
3
5
7
3T3-L1
fibroblast
Peri -/- MEF adipocyte
ATGL
B
μEq fatty acid/mg protein
3
2
1
0
**
μmol glycerol/mg protein
0.3
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forskolin
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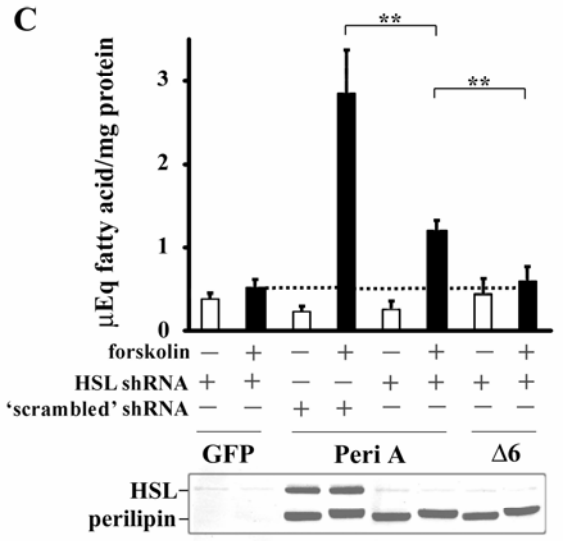
GFP
Peri A
perilipin
ATGL

Fig. 3


C
µEq fatty acid/mg protein
0
1
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**
forskolin − + − + − + − +
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GFP
Peri A
Δ6
HSL
perilipin

Fig. 4

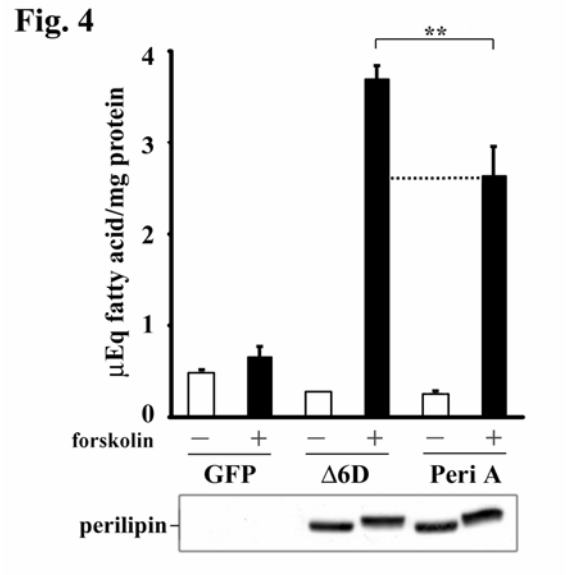
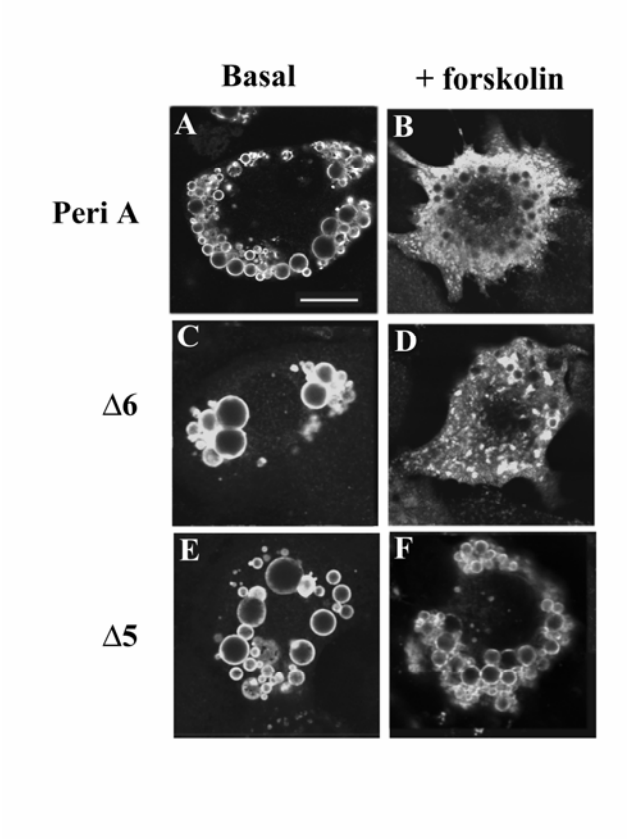
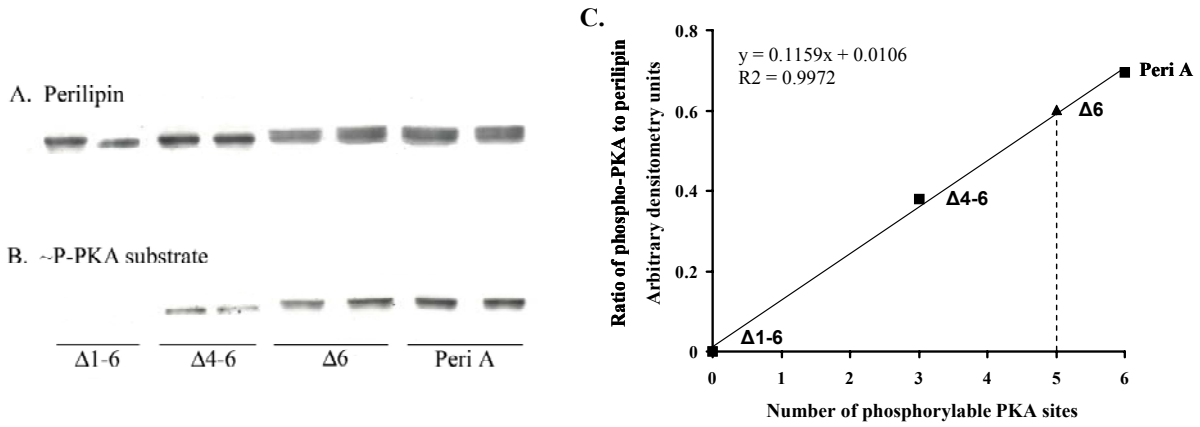

**
4
3
2
1
0
μEq fatty acid/mg protein
forskolin
–
+
–
+
–
+
GFP
Δ6D
Peri A
perilipin

Fig. 5





Supplemental Fig. 1. Standard curve of phospho-PKA immunoreactivity as a function of phosphorylable perilipin PKA sites. Lysates from Peri -/- MEFs transduced with various perilipin constructs that had been stimulated with forskolin for 20 minutes were collected. Samples were subjected to SDS-PAGE followed by immunoblotting with an antibody for anti-perilipin (A) or an antibody to PKA phospho-serine epitopes (B; Cell Signaling). Using these blots, a relative phosphorylation value for each sample was calculated as the densitometric value for phospho-PKA normalized to total perilipin. The values for Δ1-6, Δ4-6 and Peri A were used to generate a standard curve encompassing 0, 3, and 6 phosphorylated PKA sites, respectively (C). The value for Δ6 was then fit to the curve and is consistent with 5 PKA sites being phosphorylated (dotted line) indicating that mutation of serine 517 does not impair phosphorylation of other PKA sites.