Agonism with the omega-3 fatty acids α-linolenic acid and docosahexaenoic acid mediates phosphorylation of both the short and long isoforms of the human GPR120 receptor

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ABSTRACT

The newly discovered G protein-coupled receptor GPR120 has recently been shown to stimulate secretion of the gut hormones glucagon-like peptide-1 and cholecystokinin upon binding of free fatty acids, thrusting it to the forefront of drug discovery efforts for treatment of type 2 diabetes as well as satiety and obesity. Although sequences for two alternative splice variants of the human GPR120 receptor have been reported, there have been no studies which directly compare the signaling of these isoforms. We have identified an additional 16 amino acid gap containing four phospho-labile serine/threonine residues which is localized to the third intracellular loop of the GPR120-long (GPR120-L) isoform. Based on this finding, we hypothesized that the agonist-stimulated phosphorylation profiles of this isoform would be distinct from that of the short isoform (GPR120-S). Using a clonal HEK293 cell model, we examined agonist-mediated phosphorylation of GPR120-S and GPR120-L with the omega-3 fatty acids α-linolenic acid (ALA) and docosahexaenoic acid (DHA). Our results show rapid phosphorylation of both isoforms following agonism by either ALA or DHA. Moreover, we show no significant difference in the degree or rate of phosphorylation of both isoforms upon agonism with either ALA or DHA, suggesting that the additional gap in the longer variant is not phosphorylated. Importantly, our results demonstrate that the shorter variant exhibits significantly more pronounced basal phosphorylation in the absence of agonist, suggesting that the additional gap in the long variant may contribute to masking of constitutive phosphorylation sites. These are the first results which demonstrate specific phosphorylation of GPR120 isoforms upon agonism by free fatty acids and the first which distinguish the phosphorylation profiles of the two GPR120 isoforms.

1. Introduction

GPR120 is a recently discovered G protein-coupled receptor (GPCR) that was identified as a member of the rhodopsin-like GPCRs based on database mining and phylogenetic analysis [1]. Since its discovery, GPR120 has become an attractive target for treatment of type 2 diabetes due to its ability to stimulate the release of the insulin secretagogue glucagon-like peptide-1 from secretory L-cells of the intestinal lumen [2–3]. Stimulation of GPR120 has also been shown to elicit secretion of cholecystokinin *in vivo* as well as in mouse intestinal enteroendocrine cells [4], suggesting that GPR120 may have a broad role in regulating secretion of intestinal peptides.

While it has been demonstrated that GPR120 is agonized by long chained free fatty acids (FFA), which include the omega-3 fatty acids (Ω3FA), the molecular aspects involved in GPR120 signaling remain elusive. Interestingly, while the rat and mouse GPR120 genes are comprised of a single 1086 nucleotide sequence which encodes a protein of 361 amino acids, the human GPR120 gene can be alternatively spliced yielding a 361 amino acid protein (GPR120 Short, GPR120-S) and a distinct longer isoform of 377 amino acids which contains an extra exon (GPR120 Long, GPR120-L) [2–3]. Surprisingly, a recent study using a non-human primate (Cynomolgus monkey) model revealed presence of only the shorter 361 amino acid protein which was 97.5% homologous to human GPR120-S, suggesting that GPR120-L is specific to humans [5].

In this study, we examined the sequence differences between the two human GPR120 isoforms and note an additional 16 amino acid gap encoded for in GPR120-L. Importantly, we identify this addition to be localized to the third intracellular loop, a receptor domain that is critical to both G protein-coupling as well as ago-

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nist-induced receptor phosphorylation and subsequent desensitization. Moreover, this additional gap consists of four phospho-labile serine/threonine residues, suggesting that GPR120-S and GPR120-L could have distinct agonist-stimulated phosphorylation profiles. Herein, we utilize a human embryonic kidney clonal cell model to assess whether agonism of cloned human GPR120-S and GPR120-L with the Ω3FA α-linolenic acid (ALA) and docosahexaenoic acid (DHA) would facilitate receptor phosphorylation. We also examined the phosphorylation profiles of both human GPR120 isoforms in response to each Ω3FA to determine if the additional gap in GPR120-L contributes to receptor phosphorylation.

2. Materials and methods

2.1. Cloning and FLAG-epitope tagging of human GPR120

Cloning of human GPR120-S began with reverse transcription of human colon total RNA (Applied Biosystems, Austin, TX) to complimentary DNA (cDNA) using Powerscript reverse transcriptase (Takara, Mountain View, CA) and random primers. Following their synthesis, cDNA templates were amplified for 30 cycles by polymerase chain reaction (PCR) with primers corresponding to the 5′- and 3′- ends of the published human GPR120-S sequence (NCBI ID: BC101175). The A-overhang containing amplified band corresponding to the expected 1086 bp product was excised, purified and T-A cloned into the TOPO-pcDNA3.1-V5/HIS mammalian expression vector (Invitrogen, Carlsbad, CA). Sequences were validated by nucleotide sequencing at the Georgia State University DNA Sequencing Core Facility.

The addition of the eight residue FLAG (DYKDDDDA) epitope was accomplished by PCR using specifically designed primers which included 5′-SacI and 3′-HindIII restriction sites as well as the FLAG sequence incorporated within the reverse primer. Specifically, the forward primer (5′-ACCTCCGAGCTCGCCACCATGTCCCCTGAATGCG CGCGGGCA-3′) consisted of a SacI restriction site and Kozak sequence followed by the 5′-GPR120 annealing sequence (underlined). The reverse primer (5′-ACCTCCAAGCTTTTACTTGTCGTCATCGTCCTTG TAGTCGCCAGAAATAATCGACAAGTC-3′) consisted of the GPR120 annealing sequence (underlined) and FLAG-epitope sequence (italics) followed by stop codon and HindIII restriction site. The resulting C-terminally tagged amplicon was cloned into the pcDNA 3.1 V5/His expression vector (Invitrogen) and sequences verified. C-terminally FLAG-tagged GPR120-L was obtained from Origene (Rockville, MD) and sequences verified as above.

2.2. Cell culture and transfection

Laboratory stocks of Human embryonic kidney cells (HEK-293) cells were cultured in 100 mm tissue culture plates, containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1.0% penicillin–streptomycin in a humidified atmosphere of air:CO_2 (95:5%) at 37 °C. Transient transfection of FLAG-epitope tagged GPR120 cDNAs was performed using LipoD293 reagent (Signagen Laboratories, Gaithersburg, MD) according to the manufacturer directions using 5 μg of plasmid DNA in 100 mm dishes, and all experiments were performed 2 days post-transfection.

2.3. Prediction of GPR120 topology and sequence alignment

To validate the topology of the putative cloned GPR120-S and GPR120-L sequences, we utilized the computer program TMpred [7] (http://www.ch.embnet.org/software/TMPRED_form.html) along with translations of the ORFs from the published gene sequences (NCBI ID: BC101175 and NM_181745, respectively) to predict intra- and extra- cellular domains as well as membrane-spanning regions and their orientations. Protein sequences were aligned and examined using Vector NTI 10.0 software (Invitrogen).

2.4. Immunoprecipitation and immunoblotting

Forty-eight hours following transfection with FLAG-GPR120-S or FLAG-GPR120-L, HEK293 cells were washed three times in ice cold PBS and lysed at 4 °C in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM Na_2HPO_4, pH 7.4) plus protease inhibitor cocktail for 30 min. The lysate was clarified by centrifugation at 14,000 rpm for 15 min at 4 °C, and protein concentrations were standardized using a modified-Lowry protein assay (Pierce, Rockford, IL). For immunoblotting, approximately 50 μg of total protein was electrophoresed on 10% Tris–glycine SDS–PAGE gels followed by transfer to PVDF membranes. Membranes were blocked in 3% milk in Tris-buffered saline with Tween-20 (TBST) and immunoblotted with anti-FLAG (DDK) primary antibody (Origene) followed by addition of appropriate HRP-conjugated secondary antibody and detection by enhanced chemiluminescence. Immunoprecipitation was adapted from Vicentic and colleagues [7] and optimized for GPR120. Briefly, equivalent protein concentrations of lysates (as prepared above) were diluted 3-fold in RIPA buffer without NP-40, deoxycholate and SDS and rotated end over end overnight at 4 °C with anti-FLAG M2 antibody (Sigma Aldrich, St. Louis, MO) and protein G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed three times with RIPA buffer and proteins eluted by addition of 1× Laemmli sample buffer with 2.5% β-mercaptoethanol. Equivalent aliquots of the immunoprecipitated eluate were resolved by SDS–PAGE and immunoblotted as described above.

2.5. Metabolic labeling and whole cell phosphorylation assays

Assays were performed as we have previously described for the β_2-adrenergic receptor [8], but modified for GPR120. Briefly, 48 h following transfection of HEK293 cells with FLAG-GPR120-S or FLAG-GPR120-L constructs, cells were starved of phosphate in phosphate free DMEM for 75 min and incubated with 0.2 mCi $H_3^{32}PO_4$ (Perkin Elmer, Waltham, MA) for 45 min. Cells were then stimulated with 100 μM of ALA or DHA (Nu-Chek Prep, Elysian, MN) for a time course of 1–30 min followed by lysis and immunoprecipitation, as described above. Following resolution of the immunoprecipitated phosphorylated proteins on 10% SDS–PAGE gels, the gels were dried and subjected to autoradiographic film for 1–3 days at −80 °C to detect ^{32}P incorporation. NIH ImageJ software was utilized for densitometric analysis and assays were repeated at least two times for each agonist/isoform combination. Statistical significance was analyzed by Student's t-test.

3. Results

3.1. GPR120 isoform topology

Two gene sequences for the human GPR120 receptor have been identified [2,4–5] and are distinguished by an additional 48 nucleotides in the longer sequence, which encodes for an added 16 amino acids. We examined the structural topology of the two GPR120 isoform proteins using TMpred, which predicts membrane-spanning regions and their orientations [6]. Results of this analysis demonstrated that the strongly preferred model for both sequences corresponded to the typical topology of a GPCR, with the N-terminus located extracellularly, seven hydrophobic transmem-

Table 1
Topological domains of GPR120-S and GPR120-L as predicted by TMpred.

Domain or Helix #	Orientation	Residue (from–to)		Length	
		GPR120-L	GPR120-S	GPR120-L	GPR120-S
N-Term	Out–in	1–44	1–44	44	44
I	Out–in	45–65	45–65	21	21
II	In–out	73–98	73–98	26	26
III	Out–in	113–134	113–134	22	22
IV	In–out	156–177	156–177	22	22
V	Out–in	209–225	209–225	17	17
3rd ICL	In	226–281	226–265	56	40
VI	In–out	282–305	266–289	24	24
VII	Out–in	312–332	296–316	21	21
C-Term	In	333–377	317–361	45	45

brane domains, and an intracellular C-terminus (Table 1). Interestingly, the predicted structural sequences of all GPR120-S and GPR120-L domains were identical with the exception of the 16 amino acid addition in the long isoform which is localized to the third intracellular loop domain (Table 1, Fig. 1). Further analysis of this additional domain in GPR120-L revealed the presence of two threonine residues at positions 233 and 245 as well as two serine residues at positions 234 and 247 (Fig. 1).

3.2. Expression and detection of flag-tagged GPR120 proteins

Since serine and threonine residues within the third intracellular loop and carboxy-tails of GPCRs are targets of phosphorylation [9] and phosphorylated GPCRs are desensitized in their signaling properties, we wished to assess whether agonism of either GPR120 isoform would promote its phosphorylation. Moreover, since GPR120-L contains four additional putative phosphorylation

sites, we hypothesized that the agonist-stimulated phosphorylation profiles of this isoform would be distinct from that of the short isoform. Due to the lack of an adequate GPR120- immunoprecipitating antibody, in order to assess phosphorylation of GPR120 isoforms, we engineered FLAG-epitope tagged GPR120-S and GPR120-L constructs and expressed them in HEK293 cells. Fig. 2A demonstrates immunoblot detection of FLAG immunoreactive smears prototypical of GPCRs, at approximately 40 kDa, corresponding to the expected size of GPR120, confirming that FLAG-GPR120 constructs are expressed and detected following transient transfection. Furthermore, both FLAG-GPR120 isoforms could be immunoprecipitated with anti-FLAG M2 antibody prior to immu-
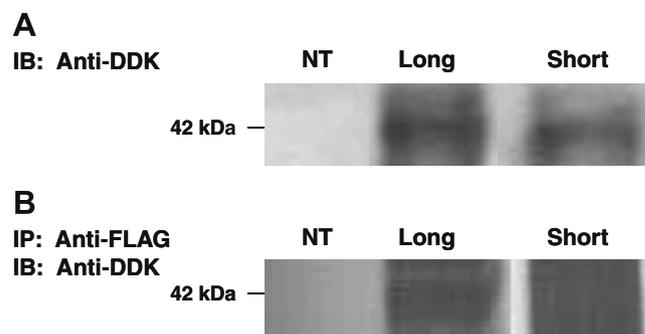
Fig. 2. Detection of FLAG-GPR120 constructs. (A) Detection of transiently transfected FLAG-GPR120-S and FLAG-GPR120-L in HEK293 cell lysates by immunoblotting reveals FLAG immunoreactive bands at ∼42 kDa, which correspond precisely to the expected molecular mass of GPR120. (B) Immunoprecipitation and immunoblotting of transiently transfected FLAG-GPR120 constructs. NT denotes non-transfected cells.
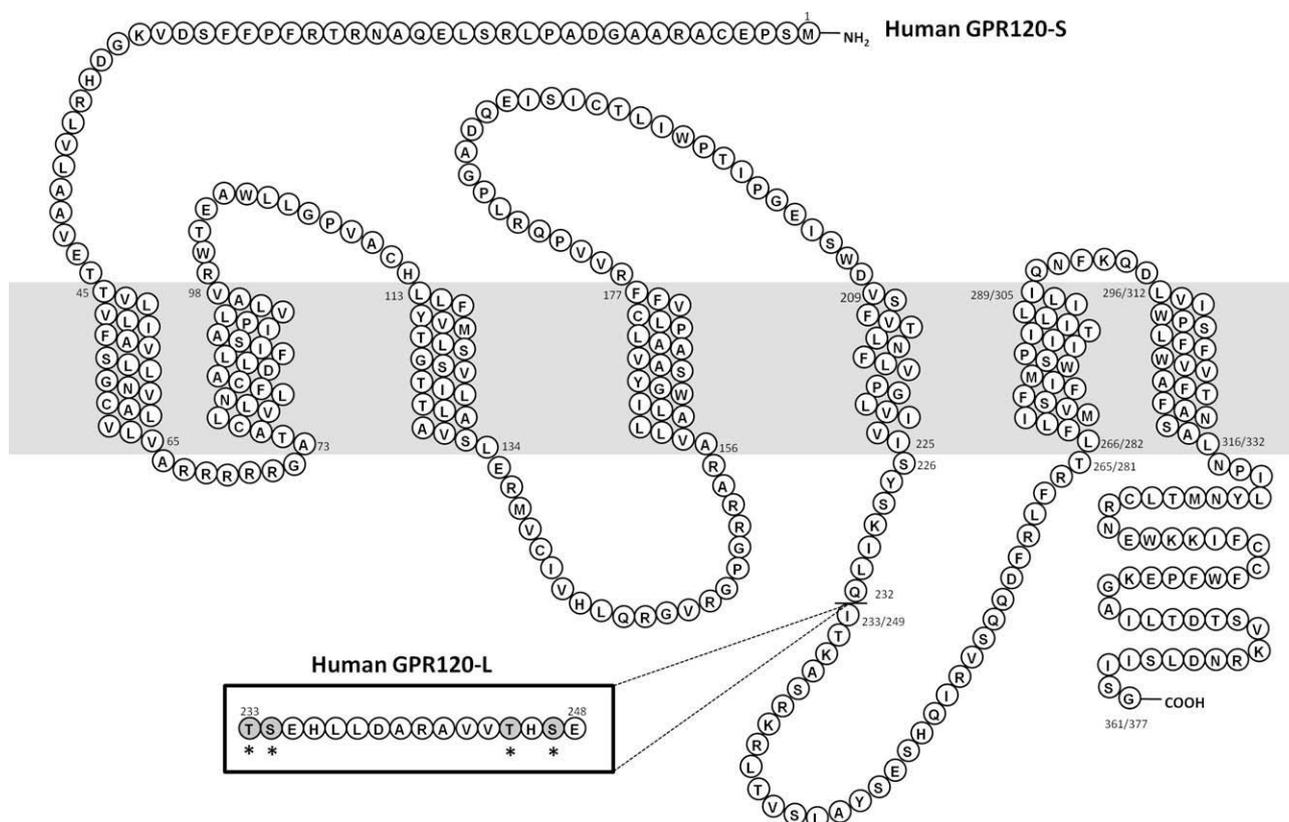
Fig. 1. Snake diagram of GPR120. The amino acid sequence of GPR120-S as predicted by TMpred. Numbers signify amino acid residues relative to initiating methionine. The insert shows the additional 16 amino acid gap afforded to GPR120-L and the four phospho-labile Ser/Thr sites are shaded and asterisked.

noblot, validating the immunoprecipitation procedure which would be used to detect receptor phosphorylation (Fig. 2B).

3.3. Whole cell phosphorylation assays

Stimulation of HEK293 cells transiently expressing GPR120-S or GPR120-L with the Ω3FA ALA and DHA resulted in rapid phosphorylation of both isoforms (Fig. 3A and B). Maximal agonist-induced phosphorylation of both isoforms peaked between 5 and 10 min following agonist addition with ALA stimulating a 159% ± 12 and 276% ± 24 maximal increase in phosphorylation (over unstimulated control) of GPR120-S and GPR120-L, respectively ($p < 0.05$) (Fig. 3C and D). Meanwhile, DHA stimulated a 276% ± 25 and 177% ± 13 maximal increase in phosphorylation (over unstimulated control) of GPR120-S and GPR120-L, respectively ($p < 0.05$ for both versus unstimulated control) (Fig. 3C and D). Notably, the approximately 1.5–2.5-fold maximal phosphate incorporation over basal upon agonism is in line with maximal phosphorylation of other class A GPCRs expressed in HEK293 cells, which presumably utilize the same endogenous pool of phosphorylative enzymes (e.g., GRKs or second messenger related kinases) [8,10–11]. These are the first results which demonstrate specific phosphorylation of GPR120 isoforms upon agonism by free fatty acids.

While Fig. 3 demonstrates that ALA and DHA can mediate specific phosphorylation of GPR120 isoforms, we also wished to assess if there were any differences in phosphorylation between the short and long GPR120 isoforms following stimulation with each FFA. Results in Fig. 4A show that stimulation of both isoforms with ALA leads to receptor phosphorylation over the time course of 30 min, with peak phosphorylation occurring between 5 and 10 min (as in Fig. 3) and a decrease in phosphate incorporation over the time course. Importantly, there was no significant difference in the phosphorylation of GPR120-S compared to GPR120-L upon stimulation with ALA throughout the time course (Fig. 4A).

Likewise, stimulation with DHA lead to rapid and robust phosphorylation of both isoforms with peak phosphorylation occurring in a similar time frame to that elicited by ALA (Fig. 4B). Similar to results with ALA, there was no significant difference in the phosphorylation of GPR120-S compared to GPR120-L upon stimulation with DHA throughout the time course. These results unambiguously demonstrate that agonism of GPR120 with FFA leads to receptor phosphorylation, and that both GPR120 isoforms are phosphorylated to the same extent over a range of stimulation times, suggesting that the additional phospho-labile Ser/Thr residues in the long isoform are not phosphorylated upon agonism.

Interestingly, our results do distinguish one significant difference in the phosphorylation profiles of the two GPR120 isoforms, specifically, that GPR120-L exhibits a lower basal level of phosphorylation compared to the shorter isoform. As seen in the representative blots in Fig. 3A and B and as demonstrated in Fig. 4C which quantifies a total of seven independent experiments, there was a 2-fold increase in GPR120-S phosphorylation (212% ± 12) in the absence of any FFA agonist compared to unstimulated GPR120-L ($p < 0.005$), suggesting that the shorter isoform is constitutively phosphorylated at one or more phospho-labile sites compared to the longer isoform. More importantly, these results suggest that the additional 16 amino acid gap within the third intracellular loop of GPR120-L somehow contributes to masking of these constitutively phosphorylated sites, which are otherwise accessible to basal phosphorylation in the shorter isoform. These are the first results which distinguish the phosphorylation profiles of the two GPR120 isoforms.

4. Discussion

A common dogma of GPCR function is that receptor signaling is rapidly abrogated via phosphorylation and subsequent functional uncoupling or internalization of the receptor [12]. In this study,
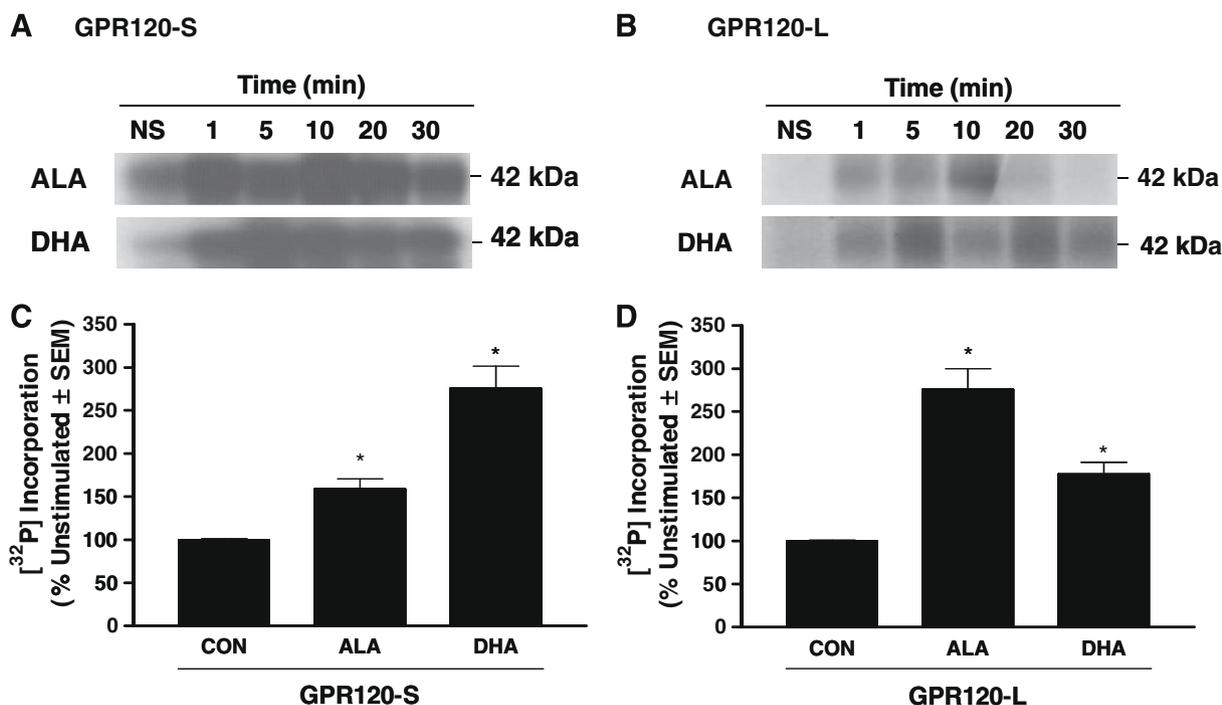

Fig. 3. Phosphorylation of GPR120 isoforms by ALA and DHA. (A and B) Agonism of GPR120-S (A) and GPR120-L (B) by ALA and DHA reveals marked increases in receptor phosphorylation over a 30 min time course with abrogation of the signal over time. NS denotes non-stimulated control. (C) Maximal phosphorylation of GPR120-S by ALA (5 min) and DHA (5 min), yielding ³²P incorporation of 159% ± 12 and 276% ± 25 above control, respectively. (D) Maximal phosphorylation of GPR120-L by ALA (10 min) and DHA (5 min) yields ³²P incorporation of 276% ± 24 and 177% ± 13 above control, respectively. Data represent mean ± SEM of at least two separate experiments. * denotes $p < 0.05$.
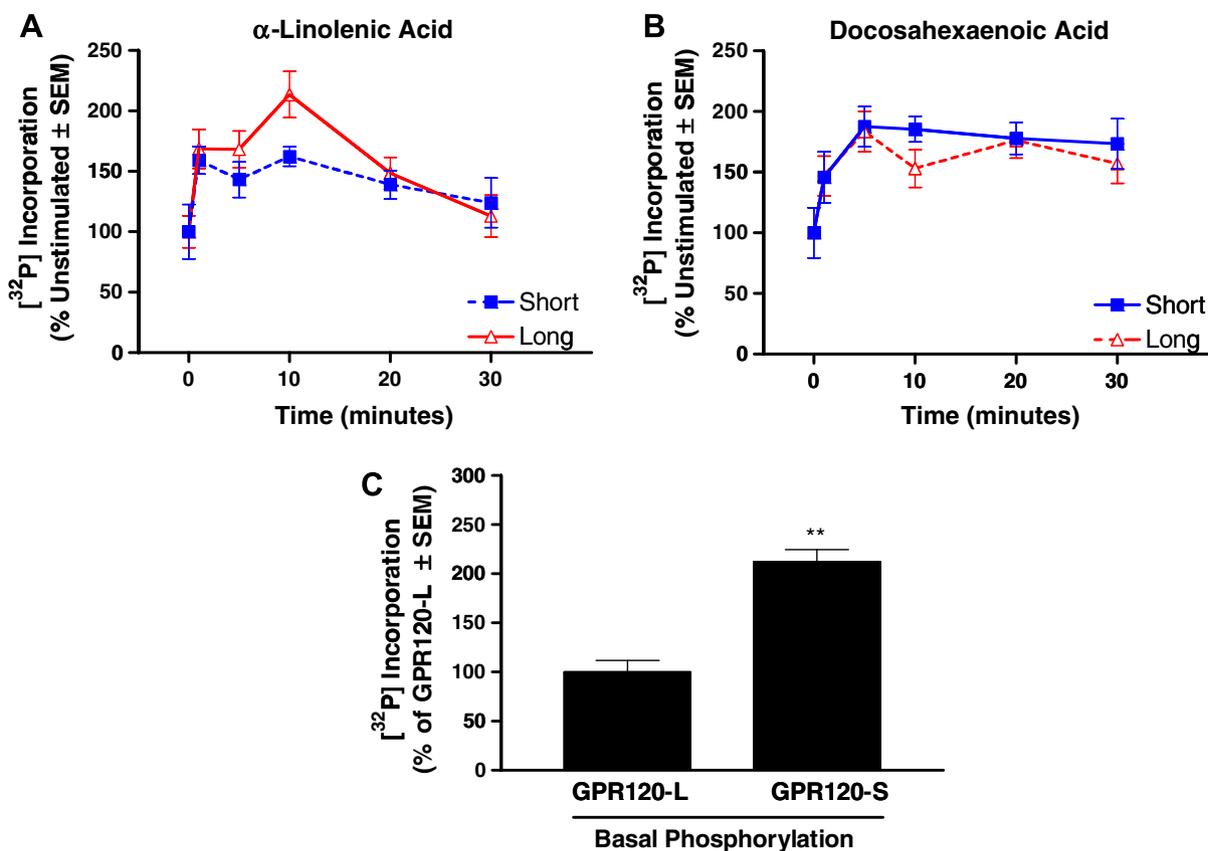
Fig. 4. Differences in phosphorylation of GPR120 isoforms by ALA and DHA. Agonism of GPR120-S and GPR120-L isoforms by ALA (A) and DHA (B) reveal no significant differences in the degree or rate of phosphorylation induced by either agonist. Data represent mean ± SEM of at least two separate experiments. (C) Levels of basal phosphorylation of GPR120-S are significantly higher than that of GPR120-L (212% ± 12 of GPR120-L). Data represent pooled mean ± SEM of seven independent experiments. ** denotes $p < 0.005$.

we wished to first examine if the two GPR120 isoforms would be phosphorylated upon agonism by FFA, and our results shown here are the first to report that agonist stimulation facilitates GPR120 phosphorylation in a rapid and transient manner. In our analysis of the differences between the two human GPR120 isoforms, we identified four phospho-labile sites which are afforded to the longer isoform and hypothesized that these sites may contribute to greater agonist-induced phosphorylation in the long isoform compared to the short. While stimulation with ALA seemed to have a minimally greater effect on phosphorylation of the long isoform, this effect was statistically equivalent to phosphorylation of the short isoform and taken together with results for DHA, we demonstrate that there is no significant difference in the intensity or rate at which GPR120-S and GPR120-L are phosphorylated, at least upon stimulation with these two agonists. These results are divergent from our original hypothesis and demonstrate that the additional Ser/Thr residues in the third intracellular loop domain of GPR120-L are likely not phosphorylated.

Agonist-occupied receptors are typically homologously phosphorylated on their cytoplasmic tails or loops by the family of G protein-coupled receptor kinases (GRKs), leading to high affinity recruitment of β-arrestin partner proteins which form signaling scaffolds, some of which can lead to receptor internalization [13–15]. Meanwhile, phosphorylation of GPCR intracellular domains can also occur upon activation of heterologous receptor systems within the same cell through second messenger associated kinases such as protein kinase A (PKA) and protein kinase C (PKC), which can facilitate functional uncoupling of receptor-G protein complexes by phosphorylating serine and threonine residues [9]. Our results demonstrate a 2-fold greater phosphorylation of GPR120-S compared to the long isoform in the absence of agonist, suggest-

ing that basal phosphorylation by PKC, PKA, or another kinase may regulate GPR120 sensitization. Interestingly, the dopamine D2 receptor is another GPCR which is alternatively spliced, yielding either D2S or D2L isoforms that are distinguished based on an additional 29 amino acids within the third intracellular loop [16] and the D2L receptor has previously been shown to demonstrate decreased basal, as well as PKC-mediated, phosphorylation due to the presence of an inhibitory PKC pseudosubstrate domain [17]. The GPR120 backbone contains two second messenger kinase associated consensus phosphorylation site(s) $(R/K)_{1–2}(X)_{1–2}(S/T)$ within third intracellular loop (K^{239}RLT and R^{254}VS) and these sites could feasibly be phosphorylated heterologously by PKA/PKC linked signaling pathways. While GPR120-L isoform does not contain a notable PKC pseudosubstrate domain, it is tempting to speculate that the mere addition of this extra gap changes the conformation or alters folding of the third intracellular loop of the long isoform such that these consensus sites are phosphorylated to a lesser degree, while they are not affected by agonist-mediated phosphorylation which putatively occurs at distinct sites through GRKs. Further ongoing studies in our laboratory will examine the precise mechanisms and site(s) of phosphorylation by these enzymes.

It is interesting that our results suggest differential maximal efficacies between the two agonists for inducing phosphorylation at the two GPR120 isoforms, e.g., DHA seems to be more efficacious at phosphorylating the short receptor while ALA more so at the long (Fig. 3C and D). This effect could be due to differences in lipophilicity or structurally dependent as DHA, a 22-carbon fatty acid with six unconjugated double bonds could interact in a distinct manner with aromatic residues that make up the receptor ligand binding pocket than could ALA, which is comprised of 18 carbons

and only three unconjugated double bonds. The number of double bonds is likely critical in binding to the receptor not only due to pi-electron interactions within the ligand binding domain, but due to the ability to constrain bond rotation thereby arranging the fatty acids in variable "kinked" arrangements. The importance of chain length and degree of saturation in promoting receptor agonism and phosphorylation will be elucidated with future studies using a broader range of fatty acids which comprise diverse lengths, double bonds, and degrees of conjugation. Additionally, another effect which could contribute to the noted differences in maximal phosphorylation efficacies could be based on the ability of a given agonist to differentially regulate phosphorylation as described by Zhang and colleagues, who reported stark differences in the ability of the structural congeners morphine and etorphine to phosphorylate the mu-opioid receptor [18]. Further ongoing studies in our laboratory on the mechanisms of GPR120 phosphorylation should shed light on this difference in efficacy between these agonists.

In conclusion, we have shown here for the first time that agonism of GPR120 with FFA leads to phosphorylation of both the short and long isoforms. Importantly, our results do not detect any difference in the rate or degree of phosphorylation between the isoforms, despite the presence of four additional phospho-labile serine/threonine residues in the long isoform. Our results do show that the long isoform is phosphorylated to a lesser degree in the absence of agonism. Further studies are underway in our laboratory to examine the sites and mechanisms of homologous and heterologous phosphorylation of the GPR120 isoforms, and to elucidate the roles of GRKs, PKA, and PKC in both.

Acknowledgments

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