

Mitotic Phosphorylation of Golgi Reassembly Stacking Protein 55 by Mitogen-activated Protein Kinase ERK2

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The role of the mitogen-activated protein kinase kinase (MKK)/extracellular-activated protein kinase (ERK) pathway in mitotic Golgi disassembly is controversial, in part because Golgi-localized targets have not been identified. We observed that Golgi reassembly stacking protein 55 (GRASP55) was phosphorylated in mitotic cells and extracts, generating a mitosis-specific phospho-epitope recognized by the MPM2 mAb. This phosphorylation was prevented by mutation of ERK consensus sites in GRASP55. GRASP55 mitotic phosphorylation was significantly reduced, both in vitro and in vivo, by treatment with U0126, a potent and specific inhibitor of MKK and thus ERK activation. Furthermore, ERK2 directly phosphorylated GRASP55 on the same residues that generated the MPM2 phospho-epitope. These results are the first demonstration of GRASP55 mitotic phosphorylation and indicate that the MKK/ERK pathway directly phosphorylates the Golgi during mitosis.

INTRODUCTION

In preparation for cell division, the highly ordered stacked cisternae of the mammalian Golgi complex undergo mitotic breakdown (Roth, 1999). This breakdown is triggered by protein phosphorylation events (Nelson, 2000) that function, at least in part, to inhibit one or more trafficking steps (Lowe *et al.*, 1998b). The exact nature of the Golgi breakdown product is controversial in that it may comprise dispersed small vesicles (Jesch and Linstedt, 1998), clustered small vesicles (Shima *et al.*, 1998), or membranes that fuse with the ER (Zaal *et al.*, 1999). In any event, the disassembled Golgi is subsequently partitioned into each daughter cell where it reassembles a ribbon of stacked membranes positioned near the microtubule organizing center.

Currently, the identity of the protein kinases that mediate Golgi disassembly is controversial. Both cyclin-dependent kinase 1 (CDK), considered a master regulatory kinase in the G2/M transition (Ohi and Gould, 1999), and a kinase in the mitogen-activated protein kinase (MAPK) signaling pathway known as MAPK kinase (MKK) have been implicated in

mitotic Golgi breakdown. In its better characterized role in cell cycle entry from G₀, MKK (present as 2 isoforms, MKK1 and MKK2), becomes activated by phosphorylation and then phosphorylates and activates the MAP kinase isoforms known as extracellular signal-regulated protein kinase (ERK) 1 and ERK2. Importantly, the MKK and ERK isoforms are present in their active forms in mitotic extracts (Kuang and Ashorn, 1993) and cells (Shapiro *et al.*, 1998; Zecevic *et al.*, 1998; Tolwinski *et al.*, 1999), and mammalian fibroblasts treated with an MKK inhibitor arrest in G₂ failing to enter M-phase (Wright *et al.*, 1999). One of the first observations implicating MKK in mitosis came from an assay that measures mitotic cytosol-dependent Golgi disassembly in permeabilized normal rat kidney cells. In this assay the Golgi breakdown activity is separable from CDK1 and is blocked by MKK inactivation or MKK immunodepletion (Acharya *et al.*, 1998). In contrast, in an assay that measures mitotic cytosol-dependent disassembly of purified rat liver Golgi, breakdown is blocked by CDK inhibition or depletion but not by MKK inhibition or depletion (Lowe *et al.*, 1998a). Recent work with a different permeabilized cell assay for mitotic Golgi breakdown has suggested a possible resolution to these controversial reports in that both CDK1 and MKK were found to be required for disassembly, but each may mediate distinct steps (Kano *et al.*, 2000).

A key point, however, is that because both CDK1 and MKK are required for events in the G₂/M transition that are upstream of Golgi breakdown, elucidation of the direct roles

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Abbreviations used: CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; ERK, extracellular signal regulated protein kinase; GRASP, Golgi reassembly stacking protein; MPM2, mitotic phosphoprotein monoclonal-2; MYC, myc tag; GST, glutathione-S-transferase.

of these kinases will ultimately rest on identification of their Golgi-localized substrates and the development of specific inhibitors against these substrates. In the case of CDK1, it directly phosphorylates GM130 (Lowe *et al.*, 1998a), and mitotically phosphorylated GM130 has reduced affinity for the vesicle docking protein p115. Thus, GM130 phosphorylation could, in principle, block docking of transport vesicles and cause Golgi vesiculation (Nakamura *et al.*, 1997). Nevertheless, other events must also take place because inhibition of the GM130/p115 interaction is not sufficient to cause Golgi breakdown (Seemann *et al.*, 2000). In the case of MKK, a Golgi localized substrate for the MKK pathway has not been identified; thus, it remains possible that the role of MKK in Golgi fragmentation is indirect.

A proteomic study to identify novel downstream targets of the MKK/ERK pathway identified Golgi reassembly stacking protein of 55 kDa (GRASP55) as a potential substrate for a MAPK (Lewis *et al.*, 2000). GRASP55 is related to GRASP65, a Golgi-localized protein that participates in the docking of transport vesicles. Inhibition of either GRASP55 or GRASP65 during cell free reassembly of previously disassembled rat liver Golgi prevents stacking (Barr *et al.*, 1997; Shorter *et al.*, 1999). GRASP65 is highly phosphorylated during mitosis (Barr *et al.*, 1997). However, to our knowledge, the phosphorylation state of GRASP55 during mitosis has not been reported. Because of the proposed direct role of MKK in Golgi disassembly during mitosis (Acharya *et al.*, 1998; Colanzi *et al.*, 2000; Kano *et al.*, 2000), we have tested the possibility that GRASP55 is a mitotic substrate of the MKK/ERK pathway.

MATERIALS AND METHODS

Cloning and Mutagenesis

Sequence corresponding to the N-terminus of human GRASP55 was cloned from a human HeLa cDNA library by PCR amplification with the use of a primer at the 5' flanking sequence of the library vector insert site and a primer corresponding to an internal human GRASP55 site as determined from a partial human GRASP55 cDNA clone, DKFZp43D156 (RZPD, Berlin). The PCR products, after generating cohesive termini with the use of T4 DNA polymerase (Stoker, 1990), were cloned into pBluescript KS (Stratagene, La Jolla, CA) and sequenced. To generate the full-length human GRASP55 cDNA, the sequence corresponding to the remaining C-terminal portion of human GRASP55 obtained from DKFZp43D156 was cloned in frame and downstream of the sequence corresponding to the N-terminus, with the use of a shared restriction site. Site-directed mutagenesis to introduce alanine codons at the required positions was carried out as described (Deng and Nickoloff, 1992) and verified by sequencing. For HeLa cell transfection, carboxyl-terminally myc-tagged GRASP55 was generated by cloning the full-length cDNA into pCS2-MT (Turner and Weintraub, 1994). Amino-terminally GST-tagged fusion constructs were generated by cloning GRASP55 cDNA into pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ).

HeLa Transfection and Labeling

Subconfluent HeLa cells were transfected with the use of calcium phosphate (Sambrook *et al.*, 1989) and 20 μ g of pCS2GRASP55-MYC plasmid DNA per 10-cm plate. The cells were split 1:2, treated with 0.5 μ g/ml nocodazole for 18–20 h (for mitotic shake-off), and harvested at 16, 48, and 72 h posttransfection, respectively. Immunofluorescence staining was carried out as described (Jesch and Linstedt, 1998) with the use of anti-myc 9e10 mouse antibody (1:200;

Evan *et al.*, 1985) and anti-giantin rabbit antibody (1:1000; Linstedt *et al.*, 1995). For 32 P labeling of mitotic cells, nocodazole-blocked transfected HeLa cells (2.7×10^6 cells) were collected by shake-off and replated in a 10-cm dish in phosphate-free DMEM supplemented with 100 IU/ml penicillin-streptomycin and 0.5 μ g/ml nocodazole. Mitotic cells were pretreated with 20 μ M U0126 or 0.2% DMSO, incubated for 30 min at 37°C, and then subsequently incubated with 0.4 mCi/ml [32 P]H₃PO₄ (Dupont NEN, Boston, MA) for 4 h at 37°C.

Cell Extract Preparation

Extracts were prepared as described (Acharya *et al.*, 1998). Mitotic HeLa cells were isolated by shake-off from subconfluent cultures grown in MEM supplemented with 10% fetal bovine serum and 0.5 μ g/ml nocodazole for 18–20 h (Knehr *et al.*, 1995). With the use of Hoechst 33258 staining, we confirmed that chromatin was intact and condensed in the collected cells, and we also confirmed that the arrested cells were viable if reincubated in the absence of nocodazole. To prepare U0126-treated extracts, mitotic shake-off cells were replated for 30 min at 37°C in the same medium supplemented with 20 μ M U0126 (Promega, Madison, WI) or 0.2% DMSO (Sigma, St. Louis, MO), the solvent for U0126. Mitotic cells were collected by centrifugation at 2000 rpm in a SA1600 rotor (Sorvall, Newtown, CT) for 5 min at 4°C, washed two times in phosphate-buffered saline (PBS), and then once in mitotic extract buffer (MEB; 15 mM PIPES [pH 7.4], 50 mM KCl, 10 mM MgCl₂, 20 mM β -mercaptoethanol, 20 mM β -glycerol phosphate, 15 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, 1 mM DTT, 0.1 mM PMSF, 0.2 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A). The cell pellet was resuspended in two packed cell volumes of MEB, swollen on ice for 10 min, and homogenized by repeated passages through a 25-gauge needle. The homogenized cells were pelleted in a tabletop ultracentrifuge in a TLA 100.2 rotor (Beckman, Fullerton, CA) at 60,000 rpm for 45 min at 4°C. The supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at -80° C. Interphase extracts were prepared from unsynchronized cells and treated exactly as for mitotic extracts. Measured protein concentration for both mitotic and interphase extracts was between 8 and 10 mg/ml.

Protein Purification and In Vitro Phosphorylation Assays

GST-GRASP55 fusion proteins were purified after induction with 0.5 mM IPTG from 1 L exponentially growing *Escherichia coli* cultures. After harvesting by centrifugation, cell pellets were frozen at -80° C, thawed on ice, and resuspended in ice-cold 20 ml TM buffer (50 mM Tris-HCl [pH 8.0], 12.5 mM MgCl₂, 1 mM EDTA, 15 mM β -mercaptoethanol) containing protease inhibitors (1 mM PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A), 200 μ g/ml lysozyme, and 1% Triton X-100. Cell lysates were disrupted by sonication and then cleared by centrifugation at $10,000 \times g$ for 30 min. Supernatants were adjusted to 40 ml with TM buffer containing 1% Triton X-100 and bound to 2 ml S-linked glutathione agarose (Sigma) for 2 h at 4°C with mixing. Beads were collected and washed by gravity with 40 ml buffer containing 50 mM Tris-HCl (pH 8.0), 1 M NaCl, then 40 ml PBS containing 1% Triton X-100, and a final wash of 40 ml TM buffer. All wash buffers contained 15 mM β -mercaptoethanol and protease inhibitors. Proteins were left attached to beads or eluted in TM containing 20% glycerol in 15 mM reduced glutathione (Sigma), snap-frozen in liquid nitrogen, and stored in aliquots at -80° C. Phosphorylation reactions with the use of cell extracts contained 25 mM Tris-HCl (pH 8.0), 6.25 mM MgCl₂, 0.5 mM EDTA, 15 mM β -mercaptoethanol, 250 μ M ATP, 2.25 μ g mitotic or interphase extract, and GST or GST-GRASP55 fusion proteins attached to glutathione agarose beads in a final volume of 20 μ l. Reaction mixtures were incubated at 30°C for 30 min. Beads were washed in ice-cold TM buffer containing protease inhibitors. In phosphatase-treated reactions, beads were further washed with 40 mM PIPES (pH 6.0) and incubated with 0.3 U potato acid phosphatase (Cooper and

King, 1986) in 40 μ l 40 mM PIPES (pH 6.0) for 20 min at 30°C. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting for MPM-2 reactivity. Phosphorylation reactions with the use of 250 ng purified recombinant kinases, prepared as described (Shapiro *et al.*, 1998), were performed under identical buffer and incubation conditions, except that soluble GST-GRASP55 fusion proteins (5 μ g) were used.

RESULTS

Our first goal was to test if GRASP55 is mitotically phosphorylated. A partial-length human cDNA encoding a protein homologous to rat GRASP55 (Shorter *et al.*, 1999) was obtained from the German sequencing consortium. PCR was used to amplify a cDNA containing the missing 5' end out of a human cDNA library. The full-length human GRASP55 cDNA obtained encodes a 452-residue protein with a high degree of sequence identity to the 454-amino acid rat GRASP55 (Figure 1). With the use of the BLOSUM62 comparison matrix the two proteins showed 89.2% identity.

Human GRASP55, tagged at its carboxy terminus with a myc-epitope (GRASP55-MYC), was transiently expressed in HeLa cells. As expected for a properly targeted Golgi protein, the expressed GRASP55-MYC colocalized with giantin (Figure 2, A and B). To analyze GRASP55 phosphorylation in these cells, we used an immunoblotting assay that used the mitotic phosphoprotein monoclonal-2 (MPM2) antibody (Davis *et al.*, 1983). MPM2 recognizes a phosphorylated S/T-P epitope in a subset of mostly unidentified mitotic phosphoproteins (Westendorf *et al.*, 1994). Of the MPM2 antigens that are identified, many play key mitotic roles regulated by phosphorylation (Kumagai and Dunphy, 1996; Renzi *et al.*, 1997). Significantly, GRASP55-MYC immunoprecipitated from nocodazole-arrested mitotic cells was strongly MPM2 reactive (Figure 2C, lane 4). In contrast, GRASP55-MYC immunoprecipitates from nonsynchronized cells or control immunoprecipitates from nonsynchronized or mitotic cells were not detectably MPM2 reactive (Figure 2C, lanes 1-3). The overall recovery of GRASP55-MYC was roughly equal from control and mitotic cells (Figure 2D). For a reason that is currently unknown, transfected GRASP55-MYC was always recovered as multiple bands. In addition, there were noticeable band shifts in mitotic cells that could in principle reflect non-MPM2 reactive mitotic phosphorylation. On the other hand, incorporation of 32 P during mitosis indicated that only a single GRASP55-MYC band was abundantly phosphorylated (see below, Figure 4B), suggesting that differential phosphorylation does not account for the other bands. Whether the major phosphorylated band contains phosphorylation sites in addition to the MPM-2 sites was not determined because the MPM-2 sites were the focus of this study. In summary, based on MPM2 reactivity and confirmed by 32 P incorporation, GRASP is mitotically phosphorylated *in vivo* and this generates one or more MPM2 epitopes.

Our next goal was to determine if the MAPK pathway plays a role in GRASP55 mitotic phosphorylation. Toward this end, we first attempted to establish *in vitro* conditions that would reproduce M-phase specific generation of the MPM2 epitope on GRASP55. Immobilized human GRASP55 fused to glutathione-S-transferase (GST) was incubated with cell extracts prepared from unsynchronized or nocodazole-arrested mitotic HeLa cells. GST-GRASP55 became highly

Human	1	<u>MG</u> SsQSV E IPGGGTEGYHVL R VQEN SP GHRAGLEPFDFI
Rat	1	<u>MG</u> SsQSV E IPGGGTEGYHVL R VQEN SP GHRAGLEPFDFI
	41	VSInGSRLNKDNDTLKDLLKANVEKPVKMLIYSSKTLELR
	41	VSI s GSRLNKDNDTLKDLLKANVEKPVKMLIYSSKTLELR
	81	EtSV TP SNLWGGQGLLVGSIRFCSFDGANENVWHVLEVES
	81	EaSV TP SNLWGGQGLLVGSIRFCSFDGANENVWHVLEVES
	121	N SP AALAGLRPHSDYIIGADTVMNESEDLFSLIETHEAKP
	121	N SP AALAGLRPHSDYIIGADTVMNESEDLFSLIETHEAKP
	161	LKLYVYNTDTDNCREV IT TPNSAWGGEGSLGCGIGYGYLH
	161	LKLYVYNTDTDNCREV IT TPNSAWGGEGSLGCGIGYGYLH
	201	RIPTRPFEEGKKISLPGQ MT G TP ITP LKDGFTqVQLSSVs
	201	RIPTRPFEEGKKISLPGQ Ma G TP ITP LKDGFTevVQLSSVn
	241	PPSL SP PGTtGiEQSLtGLSIS S TPPAVVsVLSTGVPTVP
	241	PPSL SP PGTaGvEQSLsGLSIS S APPAVSnVLSTGVPTVP
	281	LLPPQVNQSLtSVPPMNPAtTL P gL M PL P AGL P N L P N L--
	281	LLPPQVNQSLaSVPPMNPAA t LP s LM L PL s AGL P N L P N Lps
	319	--N1N L PAPHIMPGVGLPEL vn PGL P PL P S m PPRN L PGIA
	321	1sN f N L PAPHIMPGVGLPEL g k P GL P PL P S L PPRN v PGIA
	357	PL P LP S eFL P S F PL V PE s SSAAS s GE L L S SL P pts na PSD
	361	PL P m P S d FL P S F PL V PE g SSAAS a GE p L S SL P am gp -PSD
	397	PatTTAKADaaSSLTVDV l TP pta KaPTT V EDRVgDs TP v
	400	PvmTTAKAD-tSSLTVDV m SP as KvPTT V EDRVsDc TP a
	436	sEK P VSAavDANAS e SP
	438	mEK P VSAvtDANAS g as

Figure 1. Comparison of human and rat GRASP55 sequences. Alignment of the human GRASP55 and rat GRASP55 (AF110267) sequences. Capital letters indicate exact identity. The underlined region designates the amino-terminus that was cloned for this study. Potential sites of proline-directed phosphorylation are boxed. The ideal ERK consensus motif is shown in bold, and residues that were mutated to alanine are indicated with asterisks.

MPM2 reactive after reactions with mitotic extract but not in reactions with interphase extract (Figure 3A, lanes 1 and 3). As expected, MPM2 reactivity was substantially reduced in reactions where ATP was omitted or when GST-GRASP55 was subsequently treated with potato acid phosphatase (Figure 3A, lanes 2 and 4). GST alone showed no MPM2 reactivity showing that the MPM2 reactive epitope is present in GRASP55 (our unpublished results). These results indicate that GRASP55 is phosphorylated by a kinase(s) that is active specifically in mitotic cytosol.

A common approach to validate an *in vitro* phosphorylation assay is to demonstrate that the reconstituted system results in phosphorylation of the same sites that are hit *in vivo*. Therefore, we next attempted to locate the MPM2

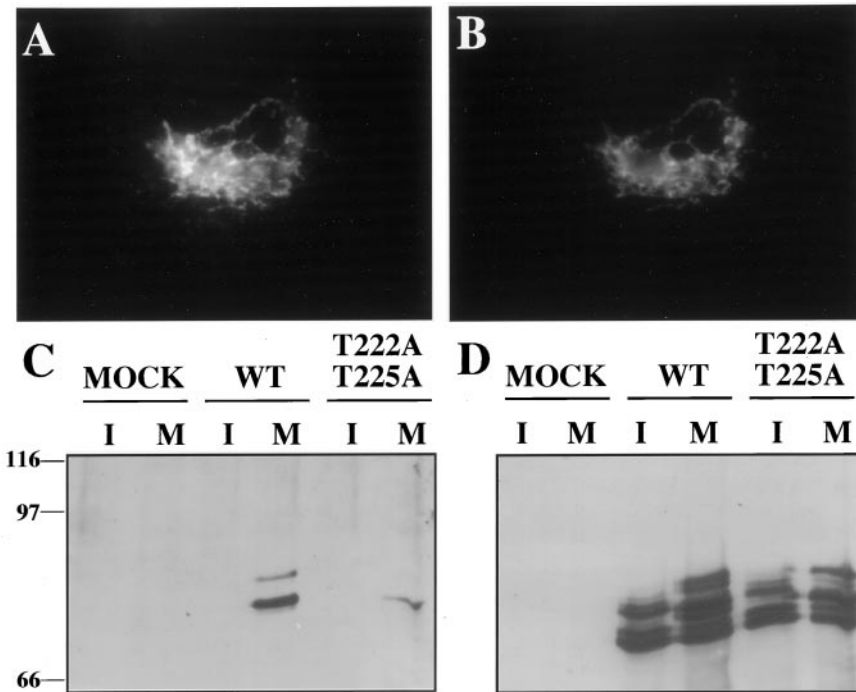


Figure 2. Human GRASP55 is a Golgi-localized MPM2 antigen. (A) HeLa cells were transfected with human GRASP55-MYC and processed for double-label immunofluorescence microscopy. The GRASP55-MYC localization pattern as indicated by anti-myc staining is shown. (B) The staining pattern of the Golgi protein giantin in the same cell. Bar, 50 μ m. (C) Unsynchronized (I) or mitotic (M) HeLa cells that were mock-transfected (lanes 1 and 2), wild-type (wt) GRASP55-MYC transfected (lanes 3 and 4), or T222A T225A GRASP55-MYC transfected (lanes 5 and 6) were subjected to immunoprecipitation with the anti-myc antibody. The resulting precipitates were analyzed for MPM2 reactivity by immunoblotting. (D) The immunoblot was re-probed with anti-myc antibody to determine GRASP55-MYC recovery. The transfected protein was recovered from both mitotic and interphase cells but only mitotic GRASP55-MYC was MPM2 reactive. As determined by densitometry and after normalization to account for the small difference in protein recovery, the MPM2 reactivity of T222A T225A GRASP55-MYC was 9% that of GRASP55-MYC.

reactive phosphorylation site in both in vitro and in vivo phosphorylated GRASP55. Eleven potential mitotic phosphorylation sites were identified in human GRASP55, and all but two of these are also present in rat GRASP55 (Figure 1, boxes). Of these, the site at T255 represented the sole ideal ERK consensus motif (Figure 1, bold). The consensus sequence for ERK is P-X-S/T-P, where P at the +1 position is favored, with secondary preference for P at the -2 position (Lewis *et al.*, 1997). We used site-directed mutagenesis to substitute alanine codons in place of T225 and T222 (a nearby site that matches the minimum ERK consensus sequence). These changes essentially abolished mitotic extract-mediated phosphorylation of GST-GRASP55 as determined by the MPM2 assay (Figure 3B). Importantly, the same substitutions in transfected GRASP55-MYC also significantly reduced its MPM2 reactivity after immunoprecipitation

from mitotic cells (Figure 2C, lane 6). This version of GRASP55-MYC was also Golgi localized, and Golgi breakdown appeared normal in the transiently transfected cells suggesting that its presence did not exert any dominant effects (our unpublished results). Although alanine substitutions at T222 and T225 were not tested individually, these results demonstrate that mitotic phosphorylation of GRASP55, both in vitro and in vivo, occurs at one or both these sites. This suggests that the same kinase mediates GRASP55 phosphorylation in both the in vitro and in vivo assays and that the identity of the kinase might be ERK.

Because our anti-ERK antibodies did not deplete ERK from the mitotic extracts, we pretreated the extracts with U0126, an inhibitor of MKK1/2 that shows little or no effect on the activities of other protein kinases (Favata *et al.*, 1998; Tolwinski *et al.*, 1999). After 30 min, active ERK was no

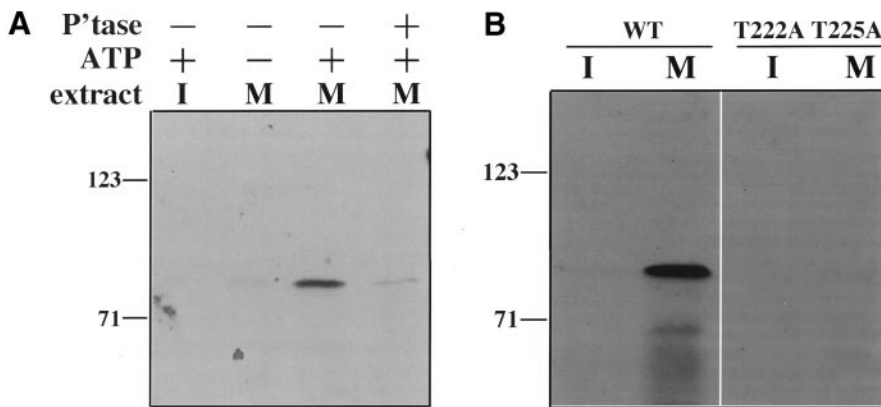


Figure 3. In vitro mitotic phosphorylation of GRASP55 on consensus ERK sites. (A) MPM2 reactivity of GST-GRASP55 was determined after an in vitro incubation with either interphase (I) or mitotic (M) HeLa cell extracts in the presence or absence of added ATP. One sample (lane 4) was treated with potato acid phosphatase before MPM2 immunoblotting. MPM2 reactivity required ATP and mitotic extract and was lost with phosphatase treatment. (B) After in vitro incubation with interphase or mitotic extracts in the presence of added ATP, the MPM2 reactivity was determined for wild-type (wt) GST-GRASP55 and a version containing T222A and T225A substitutions. Mutation of consensus ERK sites in GRASP55 blocked MPM2 reactivity.

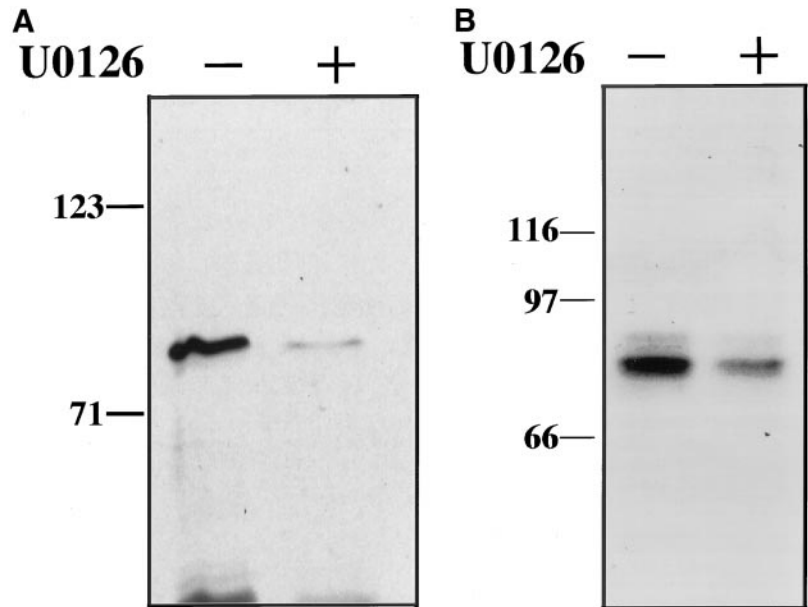


Figure 4. Inhibition of ERK activity by U0126, a specific inhibitor of MKK, inhibits GRASP55 phosphorylation. (A) MPM2 reactivity of GST-GRASP55 was determined after *in vitro* incubation with added ATP and mitotic cell extracts that had been pretreated with either 0.2% DMSO control or 20 μ M U0126 for 30 min at 37°C. (B) 32 P incorporation of transfected GRASP55-MYC recovered by immunoprecipitation from mitotic HeLa cells that were pretreated for 30 min at 37°C with either 0.2% DMSO control or 20 μ M U0126 and then incubated for 4 h with [32 P]H $_3$ PO $_4$.

longer present in mitotic HeLa cell extracts, whereas the remaining pattern of MPM2 reactivity in these extracts was not altered (our unpublished results). Therefore, U0126 had no detectable effect on other mitotically active kinases, and MKK and ERK activities are not required for the mitotic phosphorylation of most MPM2 reactive proteins. Importantly, however, GRASP55 MPM2 reactivity was significantly reduced after incubation in a U0126-treated extract compared with a nontreated control (Figure 4A). We also tested whether U0126 would inhibit 32 P incorporation into GRASP55-MYC present in transiently transfected mitotic HeLa cells. As expected mitotic cells treated with U0126 exhibited a significant reduction in GRASP55-MYC phosphorylation compared with untreated controls (Figure 4B). Equal GRASP55-MYC recovery was confirmed by silver staining (our unpublished results). Thus, as indicated by both *in vitro* and *in vivo* assays, MKK and/or ERK play a

role in GRASP55 mitotic phosphorylation. Also, turnover of GRASP55 phosphorylation site(s), presumably due to phosphatase activity, takes place in cells arrested in mitosis, and MKK and/or ERK activity is required for full rephosphorylation at these sites.

Next we tested whether ERK directly mediates GRASP55 phosphorylation. Therefore, we carried out incubations of purified GST-GRASP55 with purified and activated ERK. Significantly, GST-GRASP55 was strongly MPM2 reactive after incubation with active ERK2 (Figure 5, lane 1). Furthermore, the form of GST-GRASP55 lacking the mapped MPM2 reactive phosphorylation sites was not phosphorylated by ERK2 (Figure 5, lane 2). Two other MAPKs, p38 and much more noticeably JNK, showed some phosphorylation of GST-GRASP55 that was specific to the mapped T222 and/or T225 sites (Figure 5, lanes 3–6). These kinases, involved in the stress response pathway, exhibit overlapping substrate

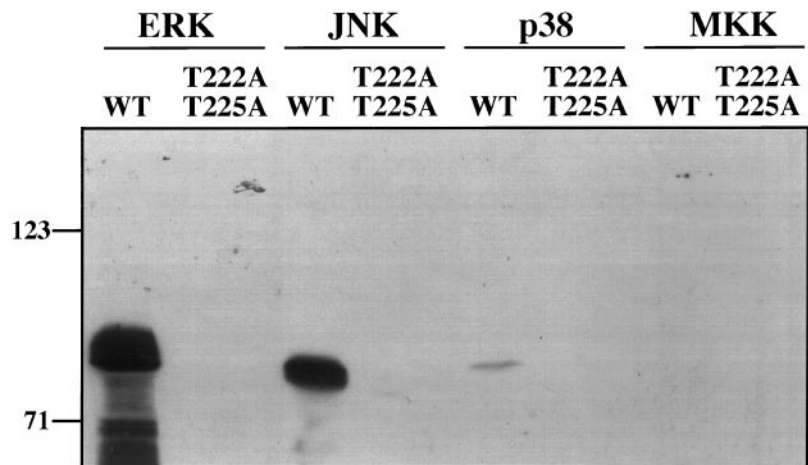


Figure 5. GRASP55 is directly phosphorylated by active MAPKs. MPM2 reactivity of equivalent amounts of wild-type (wt) or the alanine-substituted (T222A T225A) GST-GRASP55 was determined after *in vitro* incubation, 30 min at 37°C, with an ATP containing buffer and purified active ERK2, JNK, p38, or MKK1 (G7B, Δ N4/S218D/S222D). An equivalent amount of each active kinase was used in each assay.

specificity with ERK *in vitro*. p38 is not activated during mitosis (Shapiro *et al.*, 1998), and JNK is not known to be activated. In contrast to the MAPKs, a constitutively active form of MKK1, G7B: Δ N4/S218D/S222D (Mansour *et al.*, 1996), did not detectably phosphorylate either version of GST-GRASP55 (Figure 5, lanes 7 and 8). In sum, our observations demonstrate that ERK activity is required for and sufficient to mediate mitotic phosphorylation of GRASP55.

DISCUSSION

MKK activity is implicated in mitotic Golgi fragmentation (Acharya *et al.*, 1998; Colanzi *et al.*, 2000; Kano *et al.*, 2000), but without an identified Golgi substrate it is possible that its role is indirect. Our results indicate that GRASP55 is mitotically phosphorylated and that a MAPK (ERK) mediates its phosphorylation. Mitotic phosphorylation of GRASP55 in both *in vitro* and *in vivo* assays generated the MPM2 phospho-epitope. To date, seven protein kinases have been shown to generate this phospho-epitope *in vitro* including ERK (Kuang and Ashorn, 1993; Taagepera *et al.*, 1994; Kumagai and Dunphy, 1996; Renzi *et al.*, 1997; Escargueil *et al.*, 2000). GRASP55 contains several proline-directed sites for ERK phosphorylation, of which one (T225) matches the P-X-S/T-P consensus sequence and mutation of this, and an adjacent site, significantly blocked GRASP55 MPM2 reactivity. In addition, treatment of mitotic cell extracts with the MKK inhibitor U0126 produced undetectable active ERK and loss of GRASP55 MPM2 reactivity. Also, treatment of intact mitotic cells with U0126 significantly reduced 32 P incorporation into GRASP55. This result indicated that non-MPM2 reactive phosphorylation sites, if present in GRASP55, are either minor or also MKK/ERK dependent. Finally, purified active ERK2 directly phosphorylated GRASP55, and this required the same sites that generated MPM2 reactivity in mitotic cells and extracts.

It was previously demonstrated that MKK itself is mitotically phosphorylated, leading to the suggestion that this might generate an altered substrate specificity, allowing MKK to mediate Golgi breakdown in an ERK-independent manner (Colanzi *et al.*, 2000). Our observations not only indicate that the MAPK pathway directly phosphorylates a Golgi-localized substrate but also that the pathway appears to conform to a mechanism in which MKK phosphorylates and activates ERK and ERK phosphorylates the downstream target(s). Interestingly, the relevant ERK population may be Golgi-localized as anti-ERK antibody staining indicates that ERK is partially present at the Golgi (Acharya *et al.*, 1998).

GRASP55, and its homologue GRASP65, are implicated in the process by which Golgi stacks reform after mitotic breakdown (Barr *et al.*, 1997; Shorter *et al.*, 1999). Whether the GRASP proteins directly link membranes to form stacks or whether their role is upstream of the proteins that actually mediate cisternae cross-bridging remains to be determined. Nevertheless, because the Golgi disassembly/reassembly reaction during cell division is governed by reversible phosphorylation, it is reasonable to expect that the activity of proteins required for stack formation, such as the GRASP proteins, is inhibited by mitotic phosphorylation. This would allow Golgi breakdown and subsequent disinhibition by dephosphorylation would promote reassembly. On the other hand there is at present no known example of a

specific phosphorylation event that is required for Golgi breakdown. Certainly in the case of GRASP55 the role of its mitotic phosphorylation remains to be determined. It could be that if multiple kinases and multiple substrates are involved, the inhibition of any single substrate will not be sufficient to abrogate breakdown.

It is important to note that the MAPK pathway is not maximally activated at M-phase, but at other times during the cell cycle. Yet the Golgi only disassembles at M-phase, and ERK-dependent GRASP55 phosphorylation was only detected in mitotic cells or extracts. These observations indicate that the MAPK pathway that acts directly on the Golgi is M-phase specific. In this regard it is interesting to note that unlike active ERKs detected in our interphase extracts, which did not detectably phosphorylate GRASP55, purified and activated ERK2 did phosphorylate GRASP55 (Figure 5, lane 1). This suggests that cellular factors present in the extract must act in concert with ERK2 and/or GRASP55 to generate the M-phase specificity. Thus, in addition to its potential usefulness in the study of the MKK/ERK in Golgi breakdown, the work reported here on GRASP55 also provides a key reagent for dissection of the mechanism that underlies MKK/ERK M-phase specificity. The identification of Golgi-localized substrates for both CDK1 and MKK/ERK, *i.e.*, GM130 (Lowe *et al.*, 1998a) and GRASP55, respectively, is consistent with the idea that these kinases act in concert to mediate Golgi inheritance during cell division. The obvious next challenge is to demonstrate the precise role of these phosphorylation events.

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