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The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors

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Many intracellular compartments, including MHC class II-containing lysosomes [1], melanosomes [2], and phagosomes [3], move along microtubules in a bidirectional manner and in a stop-and-go fashion due to the alternating activities of a plus-end directed kinesin motor and a minus-end directed dynein-dynactin motor [4]. It is largely unclear how motor proteins are targeted specifically to different compartments. Rab GTPases recruit and/or activate several proteins involved in membrane fusion and vesicular transport [5, 6]. They associate with specific compartments after activation, which makes Rab GTPases ideal candidates for controlling motor protein binding to specific membranes. We and others [7] have identified a protein, called RILP (for Rab7-interacting lysosomal protein), that interacts with active Rab7 on late endosomes and lysosomes. Here we show that RILP prevents further cycling of Rab7. RILP expression induces the recruitment of functional dynein-dynactin motor complexes to Rab7-containing late endosomes and lysosomes. Consequently, these compartments are transported by these motors toward the minus end of microtubules, effectively inhibiting their transport toward the cell periphery. This signaling cascade may be responsible for timed and selective dynein motor recruitment onto late endosomes and lysosomes.

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Results and discussion

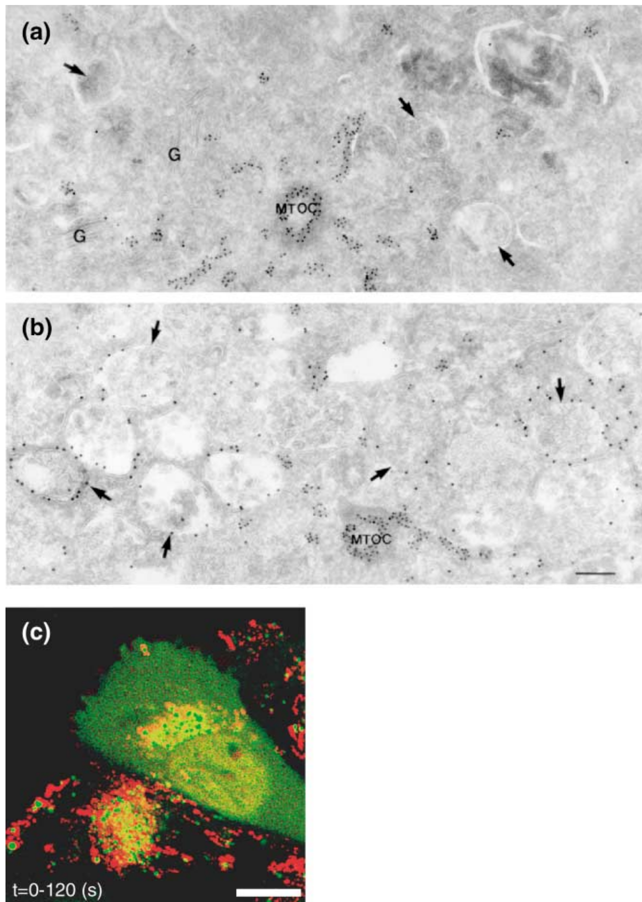
RILP requires GTP-Rab7 for clustering of late endosomes and lysosomes around the MTOC

Rab7 specifically associates with late endosomal/lysosomal compartments [8–11] and might thus regulate motor protein recruitment to these compartments. In order to identify Rab7 binding proteins involved in this process, an Epstein-Barr virus-transformed human B lymphocyte cDNA library was screened by yeast two-hybrid assay. A protein was isolated that specifically interacted with active, GTP bound Rab7Q67L but not with inactive, GDP bound Rab7T23N. The same protein (called RILP) was isolated by Cantalupo et al. [7]. When overexpressed by nuclear microinjection of cDNA in Mel JuSo cells expressing MHC class II-GFP, RILP induced a collapse of class II-containing lysosomal compartments (see Supplementary material available with this article online). This effect could be inhibited by coexpression of dominant-negative Rab7T22N (see Supplementary material). Expression of the C-terminal half of RILP (denoted ΔN) resulted in a phenotype opposite to that of full-length RILP; the class II-containing lysosomes were dispersed instead of clustered (see Supplementary material). In fact, expression of ΔN could prevent the action of full-length RILP, as shown by coexpression experiments (see Supplementary material). Apparently, ΔN competes with both endogenous and ectopically expressed RILP for binding to active Rab7Q67L, resulting in lysosome dispersion.

To analyze the RILP-induced lysosomal cluster in more detail, RILP was expressed in Mel JuSo cells by retroviral transduction. Cryosections of RILP-transduced cells were labeled with anti-RILP (large gold) and anti-tubulin (small gold) antibodies (Figure 1a,b). In control cells, lysosomal multivesicular bodies (MVB) are located at some distance to the MTOC (indicated by arrows in Figure 1a). In cells transduced with virus encoding RILP, slightly swollen MVB that were positive for RILP were densely clustered around the MTOC (Figure 1b).

The RILP-induced cluster of lysosomes is gradually formed within 1 hr after RILP injection (see Supplementary material), and during this process, the lysosomes still moved bidirectionally. To test whether the clustered lysosomes were still motile, cDNA encoding RILP was introduced via microinjection along with a marker. The lysosomes were visualized by LysoTracker Red [12]. Two hours after injection, the motility of the collapsed lyso-

Figure 1



RILP expression results in a collapse of lysosomes around the MTOC. The effect of RILP on the late endosomal/lysosomal distribution was analyzed by immunoelectron microscopy. **(a)** A micrograph of the MTOC region of a control cell and **(b)** the same region in a cell overexpressing RILP. Anti-tubulin labeled with 10 nm gold, and anti-RILP labeled with 15 nm gold. Golgi (G) and MTOC are indicated. Arrows indicate lysosomal multivesicular bodies (MVB). Note the tight packing of MVB around the MTOC in cells expressing RILP. The bars equal 200 nm. **(c)** Living Mel JuSo cells microinjected with cDNA encoding RILP were followed over a 120 s period at 37°C using time series (60 images at 2 s intervals) by CLSM. The microinjected cell was marked green, and lysosomes were visualized by LysoTracker Red. An injected cell and a control cell are shown. The collected images are projected followed by subtraction of the initial image. This procedure allows the visualization of movement, which is color-coded red. The projection (red) is merged onto the initial image (green). The bar equals 10 μm.

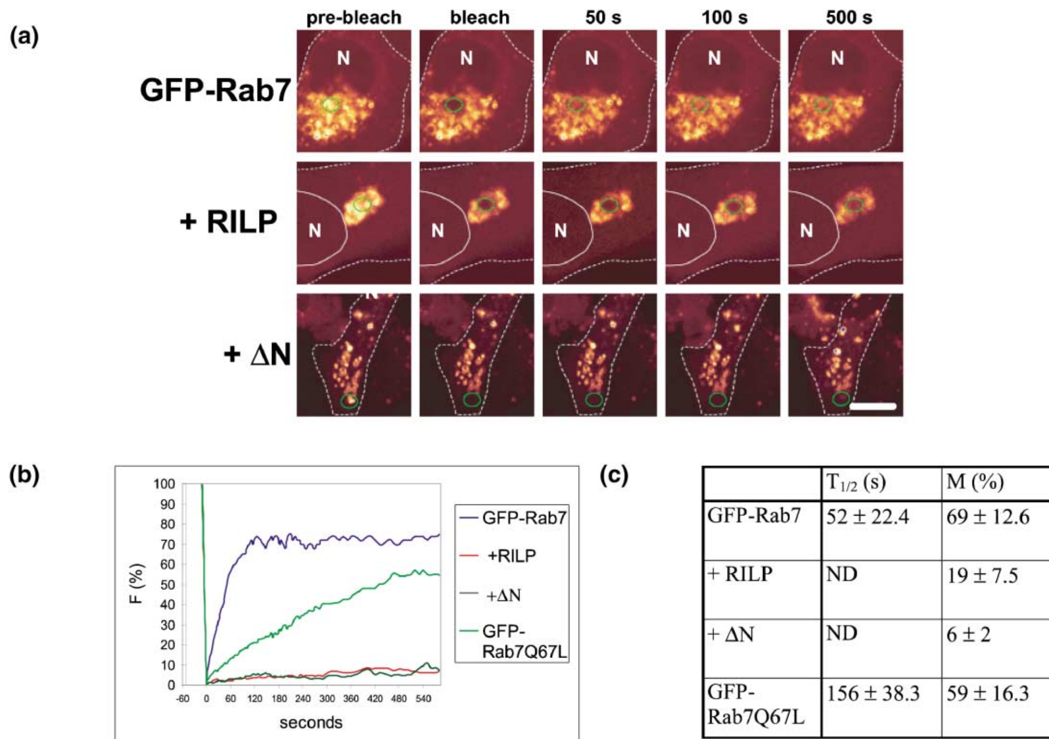
somes was assayed for 2 min by time-lapse microscopy. Collected images were projected. The first image was color-coded green, and the motility, seen in the projection as trails, was color-coded red (Figure 1c). Peripheral lysosomes were highly motile in control cells. In contrast, cells expressing RILP showed no motility of the clustered lysosomes. These results suggest that RILP expression results in a GTP bound Rab7-dependent accumulation of late endosomes and lysosomes around the MTOC and abrogates plus-end directed movement.

RILP arrests Rab7 in the vesicle bound, activated state

Rab7 cycles between a GTP bound active and a GDP bound inactive state and between the membrane and the cytosol. The GDP bound form mainly localizes to the cytoplasm, whereas the GTP bound form associates to membranes [8, 9]. It is, however, unclear where the exact GDP/GTP exchange takes place. To visualize the effect of RILP on the Rab7 cycle in vivo, Mel JuSo cells were stably transfected with both GFP-tagged wild-type Rab7 and active Rab7Q67L. Both wild-type and active Rab7 localized to late endosomal and lysosomal compartments (Figure 2a). To determine the Rab7 cycle, we performed FRAP (fluorescence recovery after photobleaching) experiments on living GFP-Rab7 and GFP-Rab7Q67L transfectants. A small portion of the fluorescent vesicles was bleached, and the recovery of fluorescence was followed by time-lapse microscopy [13]. To enable detection of bleached vesicles that lost the GFP signal, cells were incubated with LysoTracker Red (data not shown; [12]). Recovery of fluorescence can only occur when membrane bound Rab7 dissociates from the membrane upon GTP-hydrolysis and is replaced by GFP-Rab7 from the cytosol. Therefore, the fluorescence recovery represents the Rab7 cycle. The recovery of fluorescence in the bleach spot was plotted (Figure 2b), thereby allowing the determination of the recovery time ($t_{1/2}$; the time in which 50% of the fluorescence in the bleach spot was recovered) and the mobile fraction (the percentage of maximally recovered GFP-Rab7). The $t_{1/2}$ of the GFP-Rab7 cycle in vivo was approximately 52 s (Figure 2b,c). Note that there is no full recovery of the initial fluorescence; approximately 31% of the GFP-Rab7 was found in an immobile fraction. Consistently, GTP bound Rab7Q67L showed a slower recovery with a $t_{1/2}$ of 156 s (Figure 2b,c).

When RILP was expressed in GFP-Rab7 cells, clustering of the Rab7-positive lysosomes occurred within 1 hr, while expression of ΔN resulted in dispersion of lysosomes (Figure 2a, middle and bottom panels). FRAP experiments revealed that both RILP and ΔN dramatically decreased the fluorescence recovery of GFP-Rab7 on vesicles (Figure 2a–c), indicated by a markedly increased immobile fraction. An accurate $t_{1/2}$ could not be determined. Identical results were obtained for vesicles at the edge of the RILP-induced cluster as well as for single vesicles, or when RILP or ΔN was introduced in cells expressing GFP-Rab7Q67L (data not shown). Because RILP and ΔN interact with the GTP bound form of Rab7 (see Supplementary material and [7]), these data suggest that both RILP and ΔN lock Rab7 in the activated state. To further test this, GFP-Rab7 was expressed in the presence or absence of RILP in [^{32}P]orthophosphate Cos7 cells. Rab7 was isolated and analyzed by TLC (see Supplementary

Figure 2



	$T_{1/2}$ (s)	M (%)
GFP-Rab7	52 ± 22.4	69 ± 12.6
+ RILP	ND	19 ± 7.5
+ ΔN	ND	6 ± 2
GFP-Rab7Q67L	156 ± 38.3	59 ± 16.3

RILP and ΔN lock Rab7 in the vesicle bound, activated state. **(a)** Representative examples of GFP-Rab7 cells overexpressing either RILP or ΔN as indicated, shown in a glow-over/-under representation. The cells are shown before (prebleach), immediately after, and at three time points after the bleach, as indicated. The bleached area is depicted by a green circle, and the vesicles were identified by LysoTracker Red (data not shown). The nucleus (N) and the cell boundaries are indicated. The bar equals 10 μm. **(b)** Representative recovery curves of fluorescence in the bleached spot. The fluorescence (F) was related to the initial fluorescence (set at 100%) and corrected for loss of fluorescence due to bleaching and the scanning procedure. The different colors represent the different conditions tested. **(c)** Quantification of the recovery time ($t_{1/2}$) and mobile fraction (M) deduced from the recovery curves including the standard deviation. GFP-Rab7Q67L recovers three times slower than wild-type GFP-Rab7. No accurate determination can be made of the $t_{1/2}$ of RILP- and ΔN-expressing cells. Both RILP and ΔN strongly reduce the mobile fraction of GFP-Rab7.

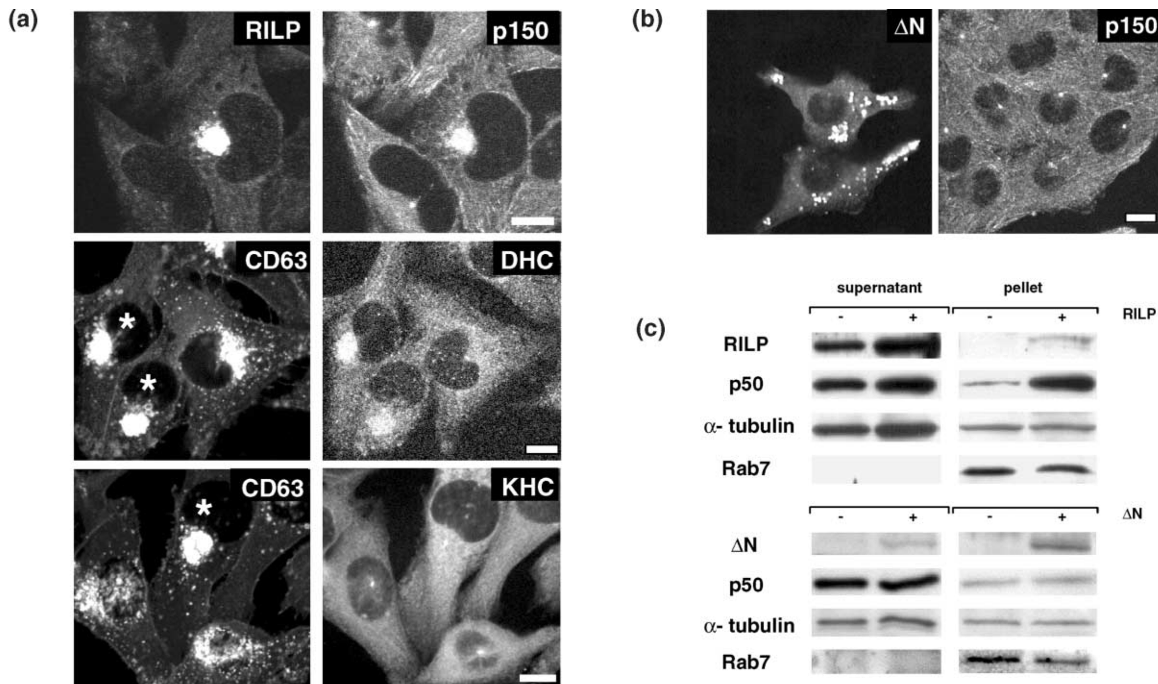
material). RILP coexpression resulted in an increase of GTP bound Rab7, which indeed suggests that RILP arrests Rab7 in the active, vesicle bound state. Various other GTPases have been described that are maintained in the GTP bound state by their effector proteins [14, 15]. Such complexes require additional proteins to inactivate the GTPase-effector interaction in order for the GTPase cycle to proceed [16]. Therefore, it is likely that the protein(s) controlling release of RILP from Rab7 is involved in the regulation of vesicle movement in the opposite direction, as illustrated by the phenotype of ΔN.

RILP induces the recruitment of functional dynein-dynactin complexes to lysosomes

The observed RILP-induced clustering could be the result of either inhibition of plus-end directed transport by kinesin or activation of minus-end directed transport by the dynein-dynactin complex. To discriminate between these possibilities, cells expressing RILP were stained for two endogenous subunits of the dynein-dynactin complex and the kinesin heavy chain (KHC). Endogenous DHC and the dynactin subunits $p150^{glued}$ (Figure 3a), p50dynamitin, and arp1 (data not shown) but not kinesin (Figure 3a) were recruited to the clustered lysosomes in cells overexpressing RILP. In cells expressing ΔN, the dispersed lysosomes did not label for endogenous $p150^{glued}$ (Figure 3b), p50dynamitin (data not shown), or kinesin (see Supplementary material).

To biochemically confirm that the RILP induced dynein-dynactin complex recruitment onto membranes, RILP or ΔN was expressed after transient retroviral transduction. Control cells and cells infected with RILP or ΔN were homogenized, and membranes were separated from the supernatant by high-speed centrifugation. Equal amounts of total protein from the fractions were analyzed by SDS-PAGE and immunoblotted (Figure 3c). RILP was mainly present in the supernatant fractions but also in the membrane fraction, whereas ΔN was predominantly present

Figure 3



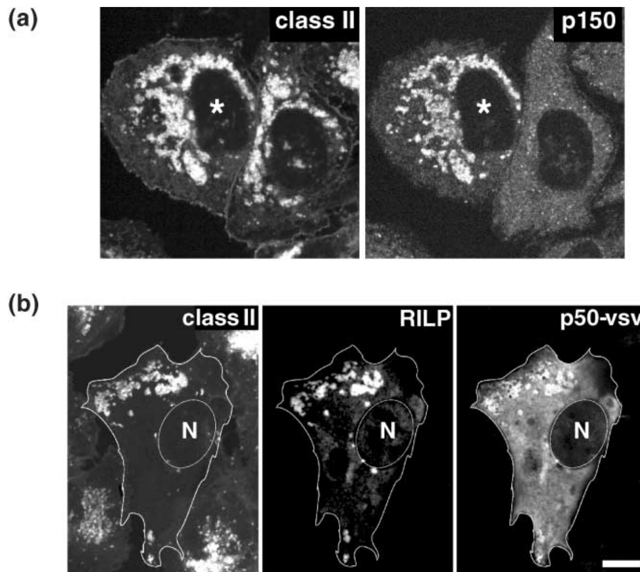
RILP induces recruitment of dynein-dynactin complexes to lysosomal compartments. **(a)** Mel JuSo cells were microinjected with cDNA encoding RILP and fixed. Injected cells were identified by RILP detection or by an injection marker. In the panels showing the localization of CD63, the RILP-expressing cells are marked by an asterisk in the nucleus. Fixed cells were labeled with anti-RILP and anti-dynactin subunit $p150^{glued}$ (top panels), anti-CD63 and anti-dynein heavy chain (DHC) (middle panels), or anti-CD63 and anti-ubiquitous kinesin heavy chain (KHC) antibodies (bottom panels). Both endogenous $p150^{glued}$ and DHC are highly enriched on the RILP-induced lysosomal clusters. The bars equal 10 μm. **(b)** Mel JuSo cells were microinjected with cDNA encoding ΔN, fixed, and stained with antibodies against ΔN (left) and the dynactin subunit $p150^{glued}$ (right). ΔN induced a lysosomal redistribution to the tip of the cell and did not cause an accumulation of the dynactin subunits on the lysosomes. The bar equals 10 μm. **(c)** Control cells and cells superinfected with virus encoding RILP or ΔN (indicated as − and +, respectively) were homogenized, and membrane and supernatant fractions were separated by high-speed centrifugation. Equal amounts of total protein were separated by 10% SDS-PAGE and analyzed by immunoblotting with the antibodies indicated. The dynein-dynactin subunit p50dynamitin is highly enriched in the membrane fraction of RILP-expressing cells, but not in the ΔN-expressing cells.

in the membrane fraction. This is consistent with the distributions observed by CLSM (see Supplementary material). RILP did not affect membrane recruitment of Rab7 and tubulin, but the dynein-dynactin subunits p50dynamitin (Figure 3c), $p150^{glued}$, and the dynein intermediate chain (DIC) (data not shown) were highly enriched in the membrane fraction of RILP-expressing cells. ΔN did not affect the distribution of any of the proteins tested. These results indicate that the N-terminal half is required for dynein-dynactin motor recruitment, whereas the C-terminal half of RILP binds to activated Rab7 on late endosomes and lysosomes.

To dissect lysosomal clustering from dynein-dynactin motor recruitment, RILP was expressed in cells treated with the microtubule-disrupting agent nocodazole (Figure 4a). As a result, the class II-containing lysosomes were dispersed but still recruited the dynactin complex (stained by $p150^{glued}$). This suggests that RILP-induced dynein motor recruitment preceded lysosomal clustering around the MTOC. To determine whether the recruited dynein-dynactin complexes mediated RILP-induced clustering of late endosomes and lysosomes, dynein function was disrupted by overexpression of p50dynamitin [17, 18]. This resulted in the relocation of lysosomes containing RILP, p50dynamitin, and class II from the perinuclear area to a peripheral location (Figure 4b). Thus, RILP induces the recruitment of functional dynein-dynactin complexes to late endosomal/lysosomal compartments resulting in minus-end directed transport and accumulation of these compartments around the MTOC.

RILP might act as a receptor for the dynein-dynactin complex. To test this, we have attempted to show a direct interaction between RILP and the motor complex by yeast two-hybrid assay, chemical crosslinking, and coimmunoprecipitation. However, none of these assays revealed a direct interaction between RILP and the subunits of the

Figure 4



RILP-induced dynein-dynactin motor recruitment precedes minus-end transport. **(a)** Mel JuSo class II-GFP cells were treated with 10 μg/ml nocodazole for 20 min before injection with cDNA encoding RILP and were subsequently cultured in the presence of nocodazole for 1 hr. Cells were fixed and stained for p150^{glued}. The cell injected with RILP is indicated by an asterisk. **(b)** A mixture of cDNA encoding RILP and an 8-fold excess of cDNA encoding vsv-tagged p50dynamitin was microinjected into Mel JuSo class II-GFP cells (left panel). p50dynamitin was visualized by anti-vsv (right panel) and RILP by anti-RILP (middle panel). The position of the plasma membrane and the nucleus (N) are indicated. The class II-containing lysosomes do not cluster in the perinuclear region, but were dispersed into the periphery. The RILP-induced collapse of the lysosomal compartments apparently requires dynein activity. The bar equals 10 μm.

dynein-dynactin motor complex. To obtain a more detailed picture of the localization of RILP with respect to the motor, we performed cryo-electron microscopy. Endogenous motor proteins were undetectable by electron microscopy; therefore, RILP was coexpressed with vsv-tagged p50dynamitin (see also Figure 4b). Whereas Rab7 and RILP often colocalized, no such colocalization was found for RILP and p50dynamitin (see Supplementary material). These data suggest, but do not exclude, that RILP does not directly interact with the dynein-dynactin motor.

How does Rab7/RILP induce selective dynein-dynactin recruitment? Most likely, RILP or RILP-regulated proteins "modify" the cytosolic phase of late endosomes and lysosomes, such that dynein-dynactin is recruited. This might be preceded by the binding of a member of the spectrin family, which acts as an intermediate structure between membranes and the dynactin subunit arp1 [19, 20] (recruited upon RILP expression; data not shown). Subsequently, the dynein-dynactin motor complex will be constructed on the spot. Whether Rab7/RILP recruit other motor proteins besides dynein-dynactin remains to be established. Other Rab family members are linked to motor proteins as well. Rab6 directly interacts with a kinesin family member [21], whereas Rab5 has been found to couple to a yet-unidentified minus-end directed motor activity [22]. Rab27 has been shown to regulate transport of melanosomes and cytolytic granules by recruitment of the actin-based motor myosin Va [23]. Thus, in addition to controlling vesicle fusion, Rab proteins appear to regulate motor protein recruitment. The control of these two processes may ensure efficient direction of vesicle fusion.

Supplementary material

Supplementary material including Materials and methods and figures is available at http://images.cellpress.com/supmat/supmatinfo.htm.

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